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Synthesis of Hyaluronan by Tumour Cells – Mechanism and Implications

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Synthesis of Hyaluronan by Tumour Cells – Mechanism and Implications

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Declaration

I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report.

Signed:

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Abstract

Hyaluronan (HA) is a glycosaminoglycan found in the extracellular matrices of epithelial and connective tissues. It has both structural and signalling functions, triggering various cell signalling cascades, which result in adhesion, migration and invasion. It has been reported that Squamous Cell Carcinomas (SCC) of the skin, oesophagus, larynx and lung, initially experience an increase in HA production. More advanced, less differentiated lesions are subject to decreased HA expression, which correlates with nodal involvement and metastasis in high-grade laryngeal and oral SCC.

The first aim of this project was to determine the mechanism increasing HA synthesis by tumours, including which signalling pathways, Hyaluronan Synthase (HAS) enzymes and cell types are responsible. The second aim was to determine what consequences this has for different cells within the tumour in terms of phenotype.

Epithelial and tumour-associated fibroblast cell lines from oral SCC were cultured in the presence of growth factors present in the tumour microenvironment (EGF, VEGF and TGF). Levels of HAS2/3 and HA production were measured using immunocytochemistry and a quantitative assay. HAS2 production was promoted in SCC epithelial cells, which corresponded with an increase in HA production.

Regarding phenotype, the addition of HA had no effect on E-Cadherin expression, a marker of epithelial cells. However, it switched on vimentin expression, a marker of mesenchymal cells, in one well-differentiated SCC epithelial line but not in the other.

These results suggest that increased HAS2 expression in transformed epithelial cells are responsible for the increase in HA, reported in SCC. Vimentin expression may indicate that

Epithelial to Mesenchymal Transition (EMT) is taking place, but this effect of HA may only occur in some SCC. Further research is needed to investigate this phenomenon.

List of Abbreviations

COMD	Tumour-associated fibroblast cell line
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
FGF-2	Fibroblast Growth Factor
GlcA	Glucuronic acid
GlcNAc	N-acetyl glucosamine
HA	Hyaluronan
HAS	Hyaluronan Synthase
ICC	Immunocytochemistry
MM1	Gingival fibroblast cell line
MMP	Matrix Metalloproteinases
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PM1	Tumour-associated epithelial cell line

ROK Rho Kinase

SCC Squamous Cell Carcinoma

TR146 Tumour-associated epithelial cell line

TYS Tumour-associated epithelial cell line

VEGF Vascular Endothelial Growth Factor

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Chapter 1 Introduction

Aims and Objectives

Squamous Cell Carcinoma (SCC) is by far the most common form of mouth cancer (Markopoulos AK, 2012). Unfortunately, it is becoming increasingly prevalent, particularly in young people, and is often detected late due to misdiagnosis and/or lack of symptoms, which has an adverse effect on prognosis (Markopoulos AK, 2012). Hyaluronan (HA) is a glycosaminoglycan found in the extracellular matrices of epithelial and connective tissues, with both structural and signalling functions. Oral SCC originates in the stratified squamous epithelium of oral mucosae, in which HA is normally abundant in the basal and spinous layers, but absent in the terminally differentiated layers (see Figure 1).

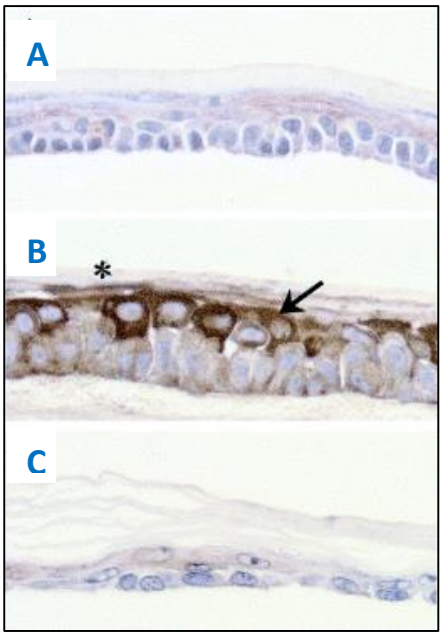


Figure 1. HA stain shows A) moderate levels of HA in the basal and spinous layers of untreated stratified squamous epithelium (keratinocyte culture), B) increased HA levels in EGF-treated culture (20ng/ml) and C) decreased HA levels in TGFβ1 treated culture (4ng/ml) (Pasonen-Seppänen., 2003).

It has been reported that SCC of the skin (Karvinen, 2003), oesophagus (Wang, 1996), larynx (Hirvikoski, 1999) and lung (Pirinen, 1998), initially experience an increase in HA production. More advanced, less differentiated lesions are subject to decreased and irregular HA expression. The reduced HA levels have been found to correlate with nodal involvement and metastasis in high-grade laryngeal and oral SCC (Hirvikoski, 1999) (Kosunen, 2004).

The aim of this project is to investigate the induction of HA synthesis in tumours and the consequences this has on cell phenotypes. Hanahan and Weinberg first emphasised the significance of 'heterotypic signalling' between normal stromal cells and the tumour cells (see Figure 2 (Hanahan D, 2000)). These interactions may take the form of growth factor production by stromal cells to facilitate tumour cell proliferation and Epithelial to Mesenchymal Transition (EMT), as well as tumour stroma formation and remodelling to facilitate tumour cell invasion and migration (Hanahan D, 2011).

Since HA is also a stromal component, it is likely that it plays a role in such heterotypic interactions. In consideration of this, this project will include a tumour cell line of oral epithelial origin (TYS), two tumour-associated fibroblast cell lines (COMD) and a normal oral fibroblast cell line as a control (MM1).

Areas of particular interest include which Hyaluronan Synthase (HAS) enzymes are responsible for the synthesis and consequent changes in HA levels, their differential regulation by growth factors and the different responses and consequent phenotypic changes in different cell types. To achieve this, the cell lines will be cultured in the presence of known HA-inducing growth factors and tested for *HAS1*/2/3 both qualitatively and quantitatively. The cells will also be tested for neoplastic markers, in particular motility and metastatic behaviours, since HA is known to be involved in tumorigenesis and motogenic activation (Ellis I, 2007).

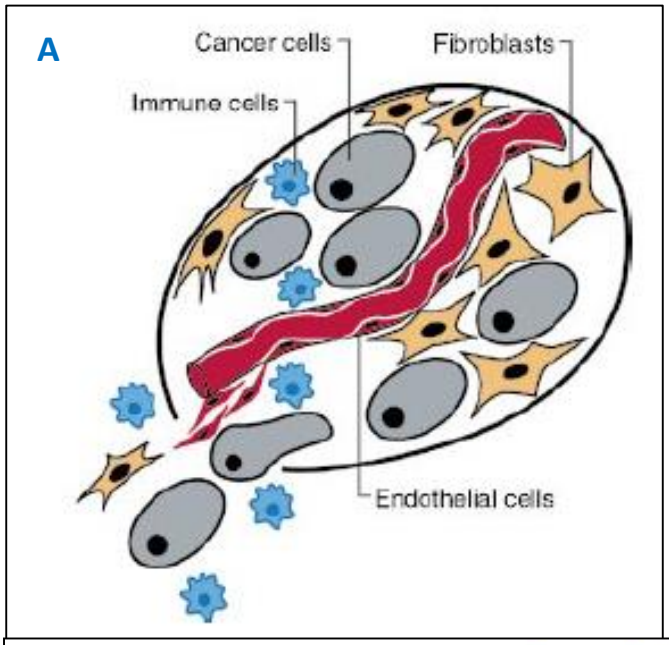
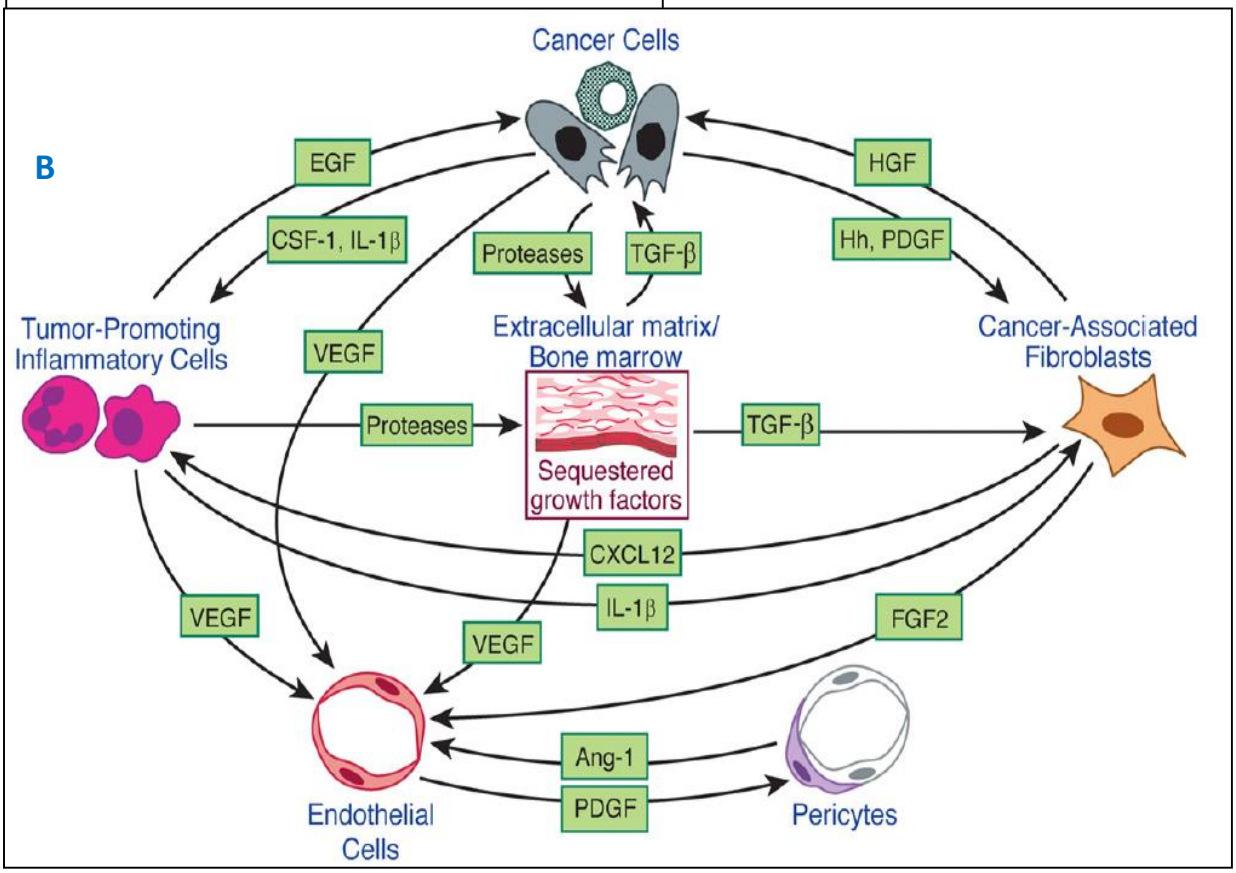


Figure 2 Tumour biology must consider the cancer cells in relation to the normal cells also present in the tumour i.e. fibroblasts, endothelial cells. A) Cells present within a tumour (Hanahan D, 2000). B) Relationship between tumour-associated cells and their signalling pathways (Hanahan D, 2011).



Hyaluronan Structure

Hyaluronan (HA) is an important and highly conserved glycosaminoglycan, ubiquitous in human tissues, with both structural and signalling roles. It is a polysaccharide chain of alternating N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) units (See Figure 3; (Linker A, n.d.)). It is unusual amongst glycosaminoglycans as it is synthesised at the plasma membrane rather than the Golgi apparatus and is therefore directly extruded into the extracellular space without sulphation or any other modification (Prehm, 1984). Nuclear Magnetic Resonance (NMR) has been used to determine that HA is a twofold helix (Atkins E, 1980), though at physiological pH in aqueous solution, the structure is likely to be locally dynamic and is now thought to form a fourfold helix on average (Almond A, 2006).

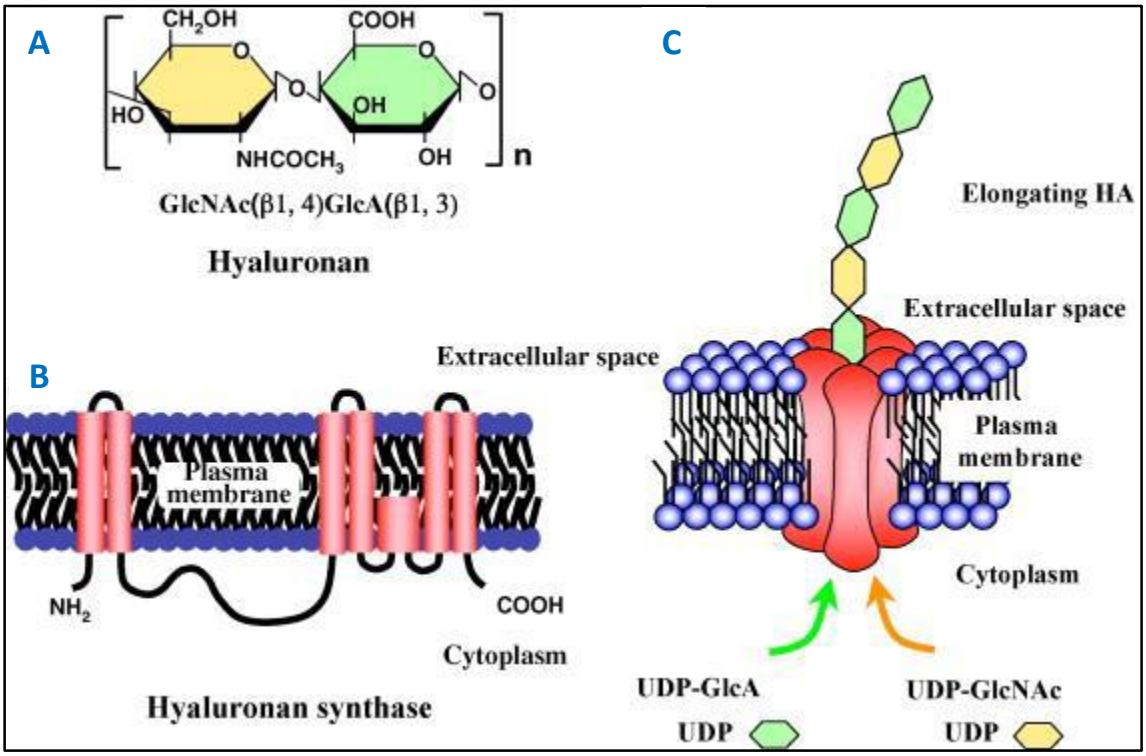


Figure 3 A) Repeating unit of Hyaluronan. B) Putative Hyaluronan Synthase structure. C) Synthesis and extrusion of Hyaluronan at Hyaluronan Synthase enzymes at the plasma membrane. (Itano N, 2008)

The structural significance of HA has long been appreciated due to its physical properties. Namely, it is deformable, has many negative charges along its length, attracting water molecules and ions, and can bind proteoglycans to form large macromolecules (Laurent TC, 1995; Heinegard D, 1974). This makes it an ideal space filling molecule, providing turgor pressure and a hydration buffer (Almond, 2007). However, it is now appreciated that HA has far more extensive roles in cell signalling and stromal regulation.

Hyaluronan Receptors

Hyaluronan receptors, also known as hyaladherins, may be found extracellularly, on cell surfaces or intracellularly (Evanko & Wight, 1999). Some of these are tissue specific while others are more widespread and may be upregulated or downregulated to control various physiological processes.

The Hyaluronan receptors LYVE-1, Stabilins and CD44, all contain a 'Link'module for binding HA (Kohda, 1996). In contrast, RHAMM contains the B(X7)B motif (also found in the Link module) in a much shorter sequence (Yang, 1994). Some of the functions of these receptors are shown in Table 1.

CD44 is the principal HA receptor found ubiquitously. It is classed as an itinerant hyaladherin as it is found both extracellularly and intracellularly. It may be considered as three major domains: an N-terminal HA binding domain, a variable middle domain and a cytoplasmic domain interacting with the cytoskeleton (Underhill, 1992). CD44 isoforms vary due to alternative splicing and post-translational modifications. The expression patterns of some of these are shown in Table 2. However, some isoforms are associated specifically with tumour cells and tumour progression (Wang, 2009). These are CD44v3, CD44v6 and CD44v10 and their nomenclature is derived from the variable exons they include (see Figure 4).

RHAMM (also designated CD168) is another itinerant hyaladherin and is also alternatively spliced but to a lesser extent (Turley, 2002; Turley E, n.d.). The truncated form is expressed specifically in response to injury, in tumour cells and in some Ras-transformed cell lines (Turley, 2002; Slevin, 2007).

Although the majority of HA binding proteins are membrane-bound receptors, some HA-binding proteins are released into the Extracellular Matrix (ECM), in particular CD44 (Knudson C, 1993; Day A, 2002). These are responsible for binding and organising stromal molecules.

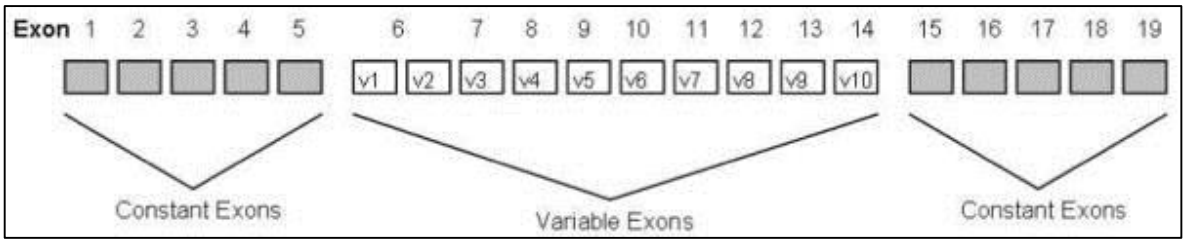


Figure 4 Exon map of CD44 gene. Note the 10 variable exons that may be alternatively spliced into variant forms. (Wang, 2009)

HA Receptor	Associated Roles
CD44	Attachment and Organisation of ECM (Day A, 2002) Cluster to attract and stimulate migration of lymphocytes during inflammation (Salmi, 2013) Cell Aggregation (Zawaideh, 1996) and Proliferation (Meran, 2011; Takano, 2009) Angiogenesis (Cao, 2006)
RHAMM	Co-receptor for integral membrane proteins Cytoskeleton remodelling for motility (Assmann, 1999) Progression past G2M boundary of cell cycle for proliferation (Assmann, 1999)
LYVE-1	Lymph specific receptor with uncertain function (Johnson, 2007); possibly HA metabolism under inflammatory conditions or cell migration Role in tumour cell adhesion (Du, 2013)
Stabilin-1	HA scavenging and regulation of cell secretory processes (Kzhyshkowska, 2006)
Stabilin-2	HA scavenging in the liver (Falkowski, 2003) Adhesion and homing of stem cells to bone marrow (Qian, 2009)

Table 1. *Roles of HA Receptors reported in the literature*

Isoform	Structural Characteristic	Location	Relative Amount
CD44H	Most common form, core protein, 37 kDa	Haemopoietic cells, leucocytes and other mesodermal cells	Major
CD44TR	Lacks cytoplasmic domain	Haemopoietic and other cell lines	Minor
CD44E (R1)	132 amino acid insert in middle domain	Epitheilia and other cells	Major
CD44E (R2)	69 amino acid insert in middle domain	Epithelia, granulocytes and mononuclear cells	Minor
CD44M	162 amino acid insert in middle domain	Malignant rat tumour cell lines	Unknown

Table 2. *CD44 Isoforms and their expression patterns. Adapted from* **(Underhill, 1992)**

Hyaluronan Signalling Pathways and Functions

Even nanogram quantities of HA have been shown to produce significant effects on cell signalling pathways and metabolism (Turley, 2002). To achieve this, CD44 and RHAMM interact with various kinase pathways as well as directly binding cytoskeleton molecules.

The signalling molecules activated by CD44 are summarised in figure 5A. Cell growth is mediated by the tyrosine kinase p185HER2 (Bourgignon, 1997), a known oncogene product. Cell migration is mediated by the tyrosine kinase C-Src, which phosphorylates the cytoskeletal protein cortactin to promote polymerisation and rearrangement of actin (Bourgignon, 2001). CD44 signalling is also mediated by Rho-like GTPases, which stimulate Rho Kinase (ROK) to phosphorylate several proteins including CD44 itself. This modification promotes CD44 binding to ankyrin, a cytoskeletal protein that links plasma membrane structures to the cytoskeleton. Ankyrin induces cytoskeleton activation as well as aiding presentation of CD44 on the plasma membrane for HA binding, promoting positive feedback (Turley, 2002). It stimulates the release of intracellular Ca^{2+}stores, promoting contraction and consequent cell motility (Wang S, 2006). It also induces degradation of the ECM to facilitate migration/invasion (Wang S, 1998). Rac1 is activated in a further signalling pathway, associated with membrane ruffling and projections for motility (Bourgignon, 2000).

Large HA chains induce quiescence and contact inhibition of cell division via CD44 and the intracellular protein Merlin (Morrison., 2001). They also suppress apoptosis (Jiang., 2005) via NF-κB to maintain epithelial integrity.

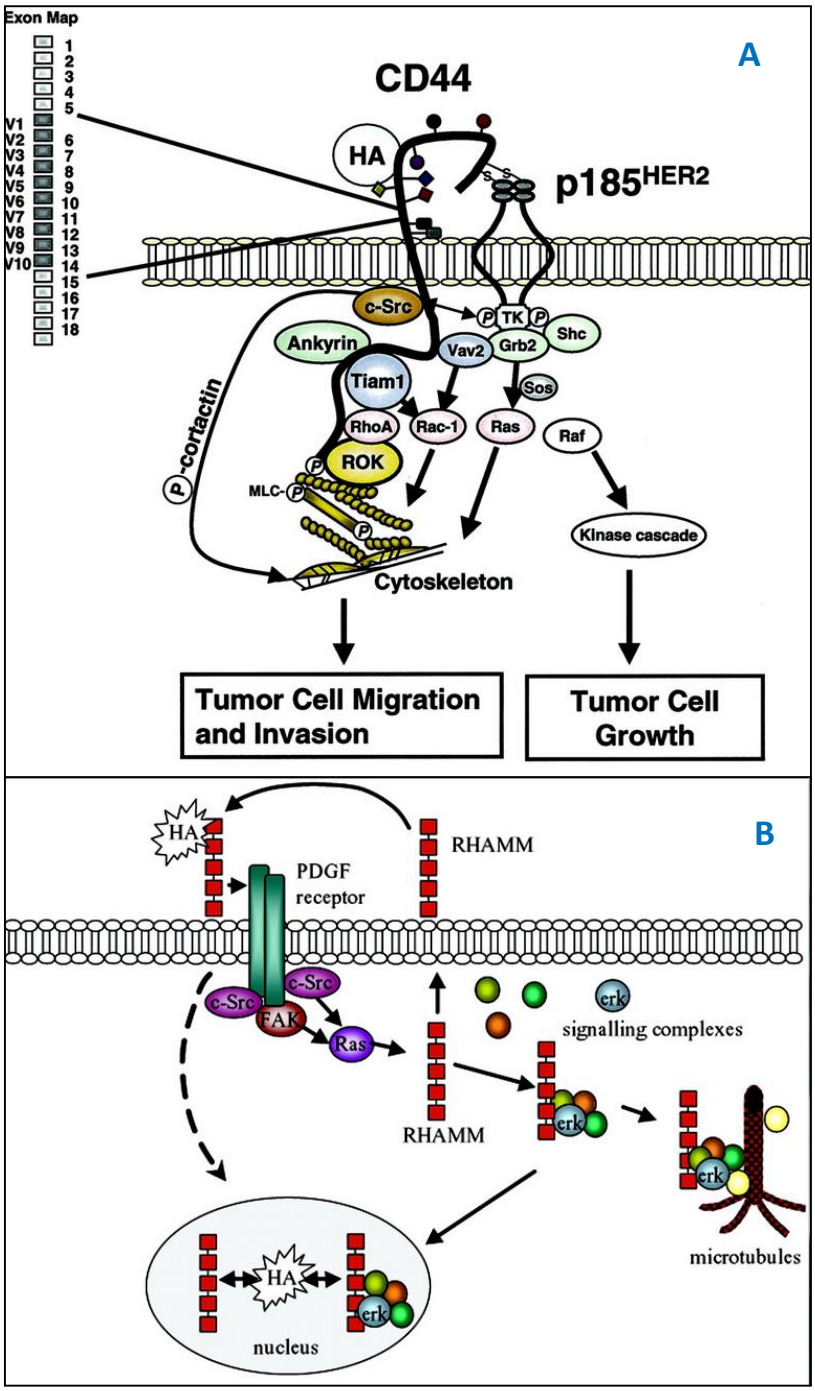


Figure 5 A) Signalling pathways associated with activation of CD44 and tumorigenesis B) Signalling pathway linking activation of RHAMM with microtubule remodelling and migration. Sourced from (Turley, 2002)

At the cell membrane, RHAMM acts as a co-receptor whose signalling is mediated by tyrosine and serine/threonine kinases (see Figure 3B). Associated tyrosine kinases include Src, Focal Adhesion Kinase (FAK), Erk and protein kinase C (PKC). Src and FAK phosphorylate cytoskeleton proteins such as cortactin and paxillin to promote rearrangement of actin and turnover of lamellae focal adhesions (Hall, 1996). RHAMM interacts with the Ras GTPase both at the cell surface and intracellularly (Hall, 1995). Intracellular RHAMM can associate with kinases, calmodulin (Lynn B, 2001) and both the actin cytoskeleton and microtubules (Assmann, 1999). In this way it links kinase pathways and directly stimulates cytoskeleton rearrangements. Interestingly it also has a role in regulating the mitotic spindle and promoting cell cycle progression past the G2M checkpoint (Assmann, 1999).

It would seem that HA signalling can have contradictory effects, both inhibiting and promoting cell cycle progression through different pathways. However, these pathways are not active simultaneously and are activated by HA molecules of different sizes. High molecular weight HA promotes cell integrity and impedes differentiation while low molecular weight HA, fragmented by hyaluronidases or oxidation by free radicals, activates signalling pathways and gene expression resulting in inflammatory responses, cell proliferation and migration. Tables 3 and 4 list some of the signalling pathways and effects stimulated by HA of different lengths.

Molecular size (saccharides)	Signalling molecules
~4	IL-12, TNFα
	Up-regulation of Fas expression
4	Erk, JNK, p38 stimulation
6	Activation of NF-κB in chondrocytes
~12	Up-regulation of PTEN in tumour cells
6-20	Inhibition of anchorage-independent growth through suppression of PI 3 kinase
~34	FAK, PI 3 kinase
Not determined	Activation of NF-κB

Table 3. *Differential signalling by HA fragments of different lengths. Adapted from* **(Stern R, 2006)**

Size (saccharides)	Function
High-molecular-mass HA>1000-5000	Suppression of angiogenesis
	Immune suppression
	Inhibition of phagocytosis
	Suppression of HA synthesis
HA fragments	
~1000	Induction of inflammatory chemokines
	Stimulation of PAI-1
	Stimulation of urokinase
10-40	Induction of CD44 cleavage
	Promotion of tumour cell migration
8-32	Stimulation of angiogenesis
	Stimulation of tumour neovascularization
~15	Suppression of smooth muscle cell proliferation
12	Endothelial cell differentiation
	Up-regulation of PTEN in tumour cells
10	Displacement of matrix HA on oocyte surface
	Displacement of proteoglycans from cell surface
6	Suppression of HA cable formation
	Induction of NO and MMPs in chondrocytes
	Induction of *HAS2* in chondrocytes
4-6	Induction of cytokine synthesis in dendritic cells
	Transcription of MMPs
4	Up-regulation of Hsp 72 expression
	Suppression of apoptosis
	Induction of chemotaxis
	Up-regulation of heat shock factor-1
	Up-regulation of Fas expression
	Suppression of proteoglycan sulphation

Table 4. *HA chain sizes and their associated functions (partial list). Adapted from* **(Stern R, 2006)**.

At the tissue level, large HA chains are space filling, anti-angiogenic (Deed, 1997), immunosuppressive and maintain stability. Conversely, smaller HA fragments are inflammatory, angiogenic (Deed, 1997) and immune-stimulatory. Immunosuppression is facilitated by the ability of high molecular weight HA to bind and coat cell surfaces, blocking ligands from binding surface receptors. Small HA fragments may be the product of degradation in pathological conditions (See "Hyaluronan Synthesis and Degradation") or they can come from a bacterial source (for example, Streptococci). It is therefore beneficial that they stimulate an immune response, binding Toll-like Receptors 2 and 4 (TLR2/4) on dendritic cells to stimulate maturation and consequent activation of T lymphocytes (Jiang, 2005).

There are problems with studying smaller HA chains (reviewed in (Turley, 2002)), including the risk of contamination and difficulties in accurately determining their size. However, the general trend reflects this divide between large HA maintaining homeostasis of the ECM and small HA inducing stress responses.

HA signalling has incredibly diverse roles in different tissues. For example, in the embryo, it is responsible for Epithelial to Mesenchymal Transition (EMT) (Camenisch, 2000) and many organogenesis processes. It is particularly crucial to chondrogenesis (Ohno, 2005). In adults, HA signalling is intrinsic to wound healing, inflammation and stromal organisation/re-organisation.

Wound healing is an excellent example of differential signalling by large and small HA molecules. Initially, there is an increase in local HA due to impaired drainage. These large HA molecules bind fibrinogen, stimulating local clot formation (Frost S, 1990). HA is later cleaved by Hyaluronidase enzymes (Hyals) and free radicals. The consequent small HA

molecules stimulate inflammation, immune reactions and angiogenesis, contributing to the ECM remodelling required in wound healing.

Hyaluronan and Tumour Cells

Tumour cells manipulate HA signalling pathways to induce many processes useful to their growth, survival and spread (i.e. proliferation, angiogenesis, and migration). By expressing specific variant forms of HA receptors, those signalling pathways favouring tumorigenesis can be promoted (see Table 5).

Certain HA oligosaccharides induce the cleavage and release of the extracytoplasmic domain of CD44 from the cell membrane. It is thought that providing this source of freely circulating CD44 fragments to higher grade tumour cells allows them to become independent of CD44-mediated regulation (Suguhara, 2003).

CD44 Variant	Differential Associations	Effect	Increased Cell Proliferation?	Increased Cell Migration?
CD44v3	VEGF	Angiogenesis	N	Y
	Matrix Metalloproteinases (e.g. MMP-9)	ECM degradation for migration		
CD44v6	MAP Kinase and Ras signalling	Proliferation	Y	N
CD44v10	Reduced affinity for HA for constitutive activation of CD44-cytoskeleton interactions	Cell motility	Y	Y

Table 5. *Tumour specific variants of CD44 and their tumorigenic effects. Adapted from* **(Wang, 2009)**

HA-CD44 interactions may also play a role in chemotherapy resistance. CD44 physically associates with the EGF receptor (EGFR), which is known to mediate oncogenic signalling and is a target of the chemotherapy drugs cisplatin, methotrexate and adriamycin. It has been shown that HA promotes CD44-EGFR association, EGFR activation and subsequent ERK1 and ERK2 phosphorylation (Wang S, 2006).

Hyaluronan Synthesis and Degradation

Three mammalian Hyaluronan Synthases (HAS) have been documented (Itano N, 2008), which synthesise HA chains by alternate addition of saccharides (See Figure 3; (Prehm, 1983)). They consist of a large central domain, making up 54-56% of the total protein, and clusters of transmembrane or membrane-associated domains. The central domain is thought to contain the catalytic regions though six discrete activities may be identified within the enzyme (Weigel PH, 2007). These include binding sites and glycosyltransferases for both GlcNAc and GlcA, a binding site for the growing HA chain and a transfer module for its extrusion.

Since chain length seems to be such a crucial factor in HA signalling, it is interesting to note that the different HAS proteins produce different length HA chains (Weigel P, 1997). HAS3 produces chains of 1 x10^6Da while HAS1 and HAS2 produce longer chains of 2 x10^5 – 2 x10^6 Da. It is also significant that the HAS proteins have different expression patterns. HAS1 is predominant in adults while HAS2 is the most significant in embryos (Tien JY, 2005). HAS3 is highly expressed in areas of inflammation and in tumour cells, which reflects the inflammatory role of shorter HA fragments (Tammi, 2011).

The dynamic nature of HA signalling makes turnover very important and actually reaches approximately 15g per day in humans (Laurent U, 1991). Turnover occurs at three levels.

Locally, cells bind, internalise and degrade HA. Stern *et al.* have produced a catabolic scheme in which HA is cleaved by successive hyaluronidase (Hyal) enzymes (Stern R, 2006). Hyal2 is a surface receptor, which makes the initial cleavage. These fragments are taken up by the cell in endosomes and delivered to acidic lysosomes where Hyal1 further degrades them to tetrasaccharides. At tissue level, HA is bound by the lymphatic vessel endothelial cell receptor LYVE-1 and delivered via lymphatic drainage and vasculature to the liver (Prevo, 2001). Here it is internalised by hepatocytes with the help of Stabilin-2 receptors and degraded by tissue-specific hyaluronidases (Weigel, 2003). Lastly, HA is degraded by free radicals under oxidative conditions, directly or indirectly in coordination with Hyals (Monzon, 2010; Soltes, 2006).

Regulation of Hyaluronan Levels

Transcription of the *HAS* genes and consequent HAS synthesis are the major regulatory systems of HA levels (Tammi, 2011). The three *HAS* genes are regulated differentially by many growth factors. For example, Transforming Growth Factor β1 (TGFβ1) upregulates *HAS1* expression while downregulating *HAS2* and *HAS3* (Oguchi T, 2004; Pasonen-Seppänen., 2003). Table 6 includes some of the reported effects of growth factors on HAS expression in different cell cultures. Although these data must be considered in context of the tissue cultures used, any of these signalling pathways could apply in more advanced, poorly differentiated tumours.

Other regulators of *HAS* transcription include corticosteroids (such as dexamethasone), which significantly downregulate *HAS* (Zhang, 2000), retinoic acid, which induces *HAS3* (Sayo T, 2013), and substrate concentrations, specifically UDP-GlcNAc (Jokela, 2011).

So far only transcriptional regulators have been named but growth factors also regulate HAS at a post-transcriptional level. All three HAS enzymes increase their activity when phosphorylated (Klewes L, 2010). This is associated with the activation of Erb-B2/extracellular signal-related kinase and the growth factors EGF, Platelet-Derived Growth Factor (PDGF) and IL-1β. Also, monoubiquitination of Lys190 is essential for HAS activity (Karousou, 2010).

Another possible stage in HAS activation may be dimerisation. It has been demonstrated that HAS2 and HAS3 can form homodimers and heterodimers (Karousou, 2010) and it has even been proposed that HAS enzymes could form part of larger multimeric complexes. However, there is little evidence for this.

Finally, recent work has revealed two antisense RNA molecules, named *HAS2 AS1* and *HAS2 AS2*, which contribute to regulation of HAS synthesis *in vitro* (Chao H, 2005) and is likely to similarly decrease HAS synthesis *in vivo*. It is complementary to the first exon of *HAS2* mRNA, including the transcription and translation initiation sites. Binding of the *HAS2* and *HAS AS1* mRNA not only blocks these processes but also targets the strand for degradation. It is therefore a rapid and effective mechanism for arresting HAS synthesis.

Growth Factor	Effect on *Has* Expression			Context of Interactions
	HAS1	*HAS2*	*HAS3*	
TGFβ$_1$	↑	↓	↓	Synovial fibroblasts, Organotypic keratinocytes, Mesothelial cells
EGF		↑	↑	Synovial fibroblasts, Organotypic keratinocytes,
PDGF	↑	↑	↑	Mesothelial cells
FGF-2	↑	↑	↑	Periodontal Ligament fibroblasts (during inflammation)
IL-1β	↑	↑	↑	Synovial fibroblasts, Skin fibroblasts
TNFα	↑	↑	↑	Synovial fibroblasts, Skin fibroblasts

Table 6. *Some known growth factor effects on Has expression from sources* **(Pasonen-Seppänen., 2003; Oguchi T, 2004; Tammi, 2011; Jacobson A, 2000; Shimabukuro, 2011; Campo G, 2006)**. *Key: ↑Upregulated; ↓Downregulated*

Possible Growth Factors Present in the Tumour Microenvironment

Transforming Growth Factor β (TGFβ1)

The TGFβ superfamily consists of several structurally and functionally linked growth factors, expressed in many embryo and adult tissues. Regulation of TGFβ activity occurs by regulation of TGFβ gene transcription, the need for activation of TGFβ and sequestration of activated TGFβ by the ECM (Massague, 1990). Cell responses to TGFβ depend on the cell type and presence of other growth factors, but TGFβ is associated with the regulation of many cell processes such as proliferation, migration, apoptosis, adhesion and angiogenesis (Jakowlew, 2006). TGFβ signalling operates predominantly via SMAD pathways (Derynck R, 2003). Ligand binding causes the two TGFβ receptors to form a complex with serine/threonine kinase activity. This differentially activates members of the R-SMAD

second messenger family, which bind the Co-SMAD (SMAD4) to translocate to the nucleus and interact with co-activators or co-repressors of gene expression. A simplified version of SMAD-mediated signalling is given in Figure 6. However, members of the SMAD pathway interact with other signalling molecules.

It was initially thought TGFβ would act simply as a tumour suppressor due to its autocrine promotion of cell cycle arrest and apoptosis in epithelial tissues (Arteaga C, 1990). There is now evidence for TGFβ as both a tumour suppressor and pro-oncogenic factor. TGFβ and SMADs interact with proteins directly involved in regulating the cell cycle such as Cyclin Dependent Kinases (CDKs) and can induce the synthesis of cyclins (Jakowlew, 2006). In many cancers, the TGFβ gene has been found to be mutated or the TGFβ receptor has been defective (Jakowlew, 2006). In this way, tumour cells can escape regulation by TGFβ and continue to proliferate uninhibited. Mutations of SMAD4 have also been associated with the progression of colorectal cancer (Xu, 2000) and the constitutive phosphorylation of SMAD2 is associated with poor prognosis of HNSCC (Xie, 2003). It is thought that SMAD4 exerts its own tumour-suppressor effect by inhibiting Ras-dependent Erk (Extracellular signal Regulated Kinase) signalling (Iglesias, 2000). When SMAD4 is inactivated, Erk signalling may become excessive and contribute to tumour progression.

The absence or alteration of TGFβ signalling may help to initiate tumorigenesis, but the presence of TGFβ signalling later takes on a pro-oncogenic role. It plays a role in stromal regulation, immune suppression and angiogenesis (Jakowlew, 2006), although its role in angiogenesis is likely to be indirect via induction of VEGF (Pertovaara, 1994). It may also induce Epithelial to Mesenchymal Transition (EMT) though only if the Erk pathway is also activated (Xie, 2004).

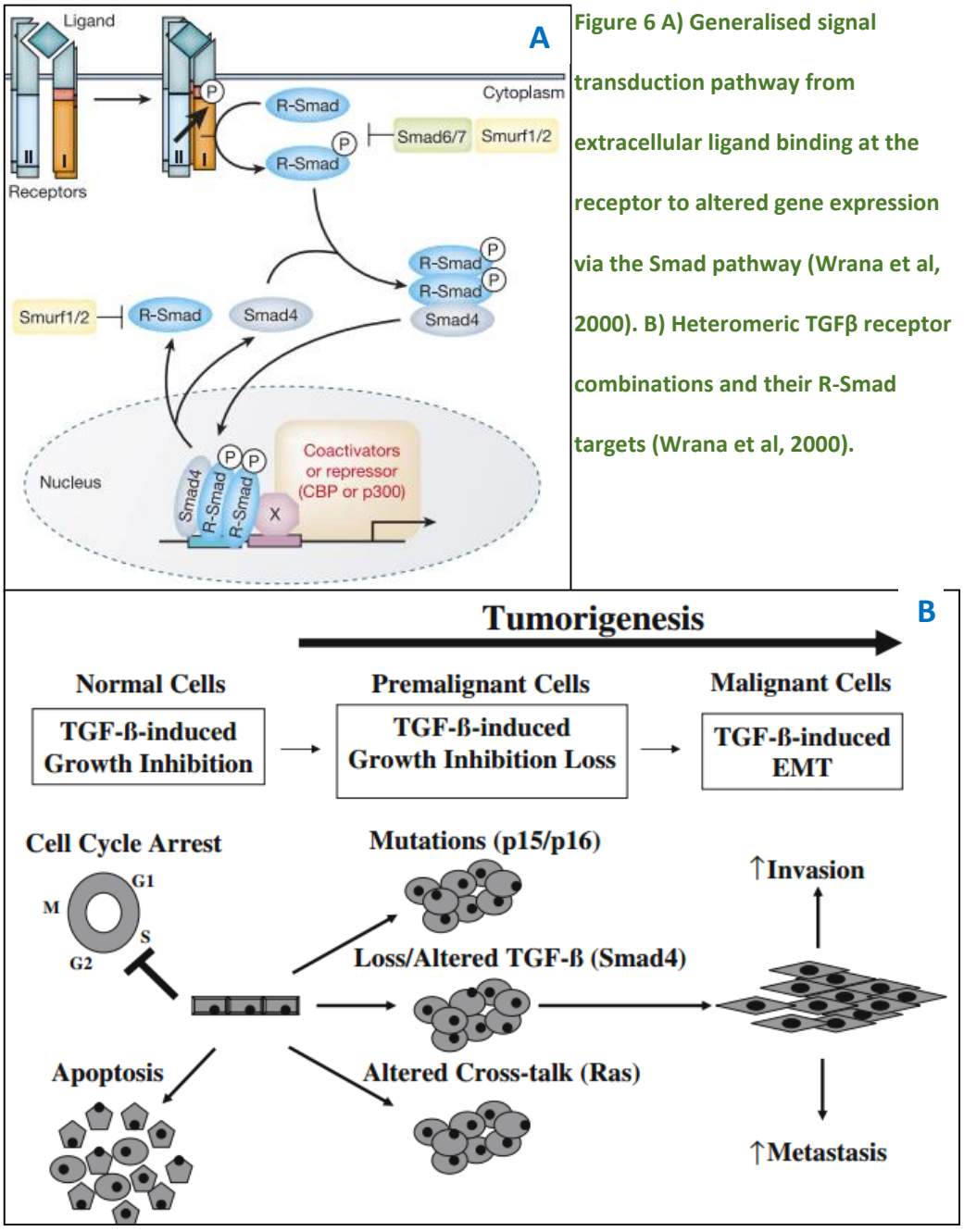


Figure 6 A) Generalised signal transduction pathway from extracellular ligand binding at the receptor to altered gene expression via the Smad pathway (Wrana et al, 2000). B) Heteromeric TGFβ receptor combinations and their R-Smad targets (Wrana et al, 2000).

Epidermal Growth Factor (EGF)

The Epidermal Growth Factor-like molecules, including TGFα, amphiregulin and of course EGF itself, are encoded by separate genes and are produced for both paracrine and autocrine signalling. This family is characterised by their ability to bind the EGF receptor (EGFR) and induce a mitogenic response, and a motif of six half-cysteine residues ($X_nCX_7CX_{2-3}GXCX_{10-13}CXCX_3YXGXRCX_4LX_n$) (Carpenter G, 1990). The EGF receptor family, also known as ErbB, consists of an extracellular ligand binding domain, a single hydrophobic anchor and tyrosine kinase cytoplasmic domain, and is similar in structure to the receptors for PDGF, FGF and Insulin-like Growth Factors (IGF) (Carpenter G, 1990). Their cytoplasmic tyrosine kinase domains are similar to that of many oncogene products. They activate multiple signalling cascades including the Phosphoinositide 3-Kinase (PI3K) pathway, RAS/ERK pathway and JAK/STAT pathway (see Figure 7). These leads to the activation of kinases, which can inactivate pro-apoptotic proteins, and transcription factors such as Nuclear Factor-κB (NF-κB) and STAT, which translocate to the nucleus to activate anti-apoptotic and cell-proliferative genes (Sethi G, 2007). *HAS2* and *HAS3* are also upregulated by EGF (Chow G, 2010).

Increased expression of EGFR and/or its ligands is a feature of most carcinomas (Zhang X, 2012; Baselga J, 2005) and some carcinoma EGFR have been found to gain ligand-independent activity (Sethi G, 2007). Overexpression of EGFR in oral cancers has been associated with malignancy and poor prognosis (Ribeiro, et al., 2014). Since EGFR signalling is known to be such a significant contributor to tumour initiation and progression, it is the target of many cancer therapies in the form of monoclonal antibodies, which bind EGFR, or protein kinase inhibitors (Zhang X, 2012).

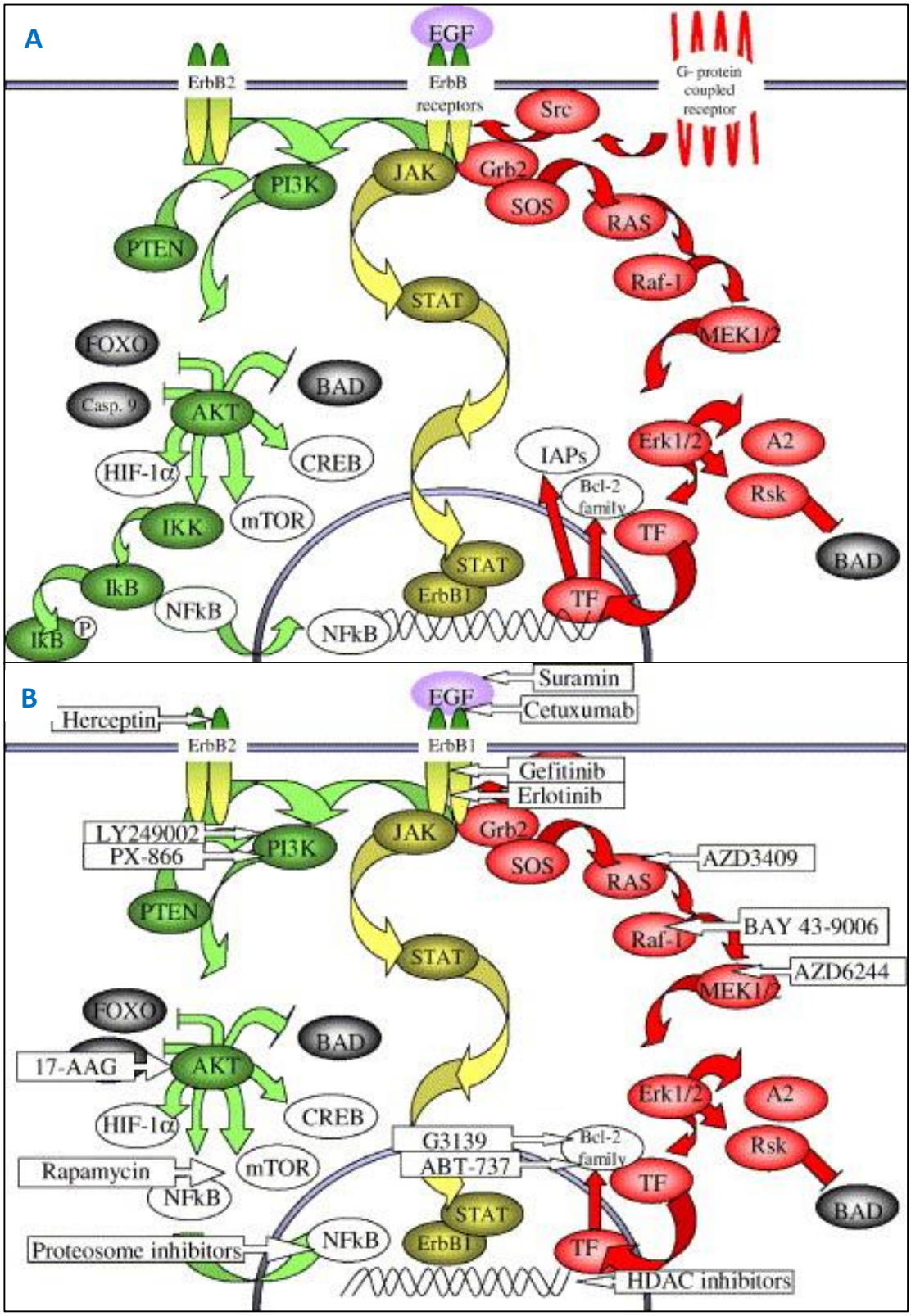


Figure 7 A) EGFR signalling pathways including the Phosphoinositide 3-Kinase Pathway (green), RAS/ERK pathway (red) and JAK/STAT pathway (yellow). These activate various anti-apoptotic proteins (white) and inactivate pro-apoptotic proteins (black). B) Targets of current cancer therapies (Henson E, 2006)

Fibroblast Growth Factor -2 (FGF-2)

FGF2 is a polypeptide growth factor with unconventional secretory protein as it can bind $PI(4,5)P_2$ directly and thereby translocate plasma membranes without the need for intracellular, membrane-bound intermediate vesicles (Nickel, 2011). FGF is involved in diverse processes including neurotrophic activity, lymphangiogenesis, stem cell differentiation, osteogenesis and tumour cell migration and invasion (Wu X, 2003).

There are 22 FGFs but the communication from stromal cells to epithelial cells is mediated by stromal FGF7/FGF10 and epithelial FGF2. The angiogenic effect of FGF2 is synergistic with signalling by PDGF-BB and VEGF (Zhang X, 2012). The receptors for PDGF (PDGFRα and PDGFRβ) are upregulated in endothelial cells in the presence of FGF-2 signalling (Cao Y, 2008). Also, PDGF-BB is able to induce FGFR-1 in vascular smooth muscle cells and sensitise them to FGF-2 (Cao Y, 2008). Regarding VEGF, the synergistic relationship takes a simpler form in that both signalling pathways involve the activation of Src tyrosine kinases. These activate focal adhesion kinase (FAK) and subsequently Mitogen-activated protein kinase (MAPK), which leads to reorganisation of the cytoskeleton actin and cell motility (Yan W, 2008).

Platelet-Derived Growth Factor (PDGF)

The PDGF family are the homo or heterodimers: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. They are bound by the receptors PDGFR-α (except PDGF-DD) and PDGFR-β (only PDGF-BB and PDGF-DD) (Heldin C, 2002). PDGFR-α is expressed during early embryonic development whereas PDGFR-β is expressed by mesenchymal cells and is upregulated during wound healing. Both receptors may therefore be of interest in tumour cells, which share characteristics with each of these cell types.

The activation of PDGFR results in homodimerisation and tyrosine kinase domain activation, which leads to activation of Src, PI3K, AKT, Ras and ERK pathways (Taylor, 2000). These are known to promote cell survival and proliferation.

Vascular Endothelial Growth Factor (VEGF)

VEGF is a family of five soluble heparin-binding polypeptide growth factors, promoting endothelial cell migration (Ferrara, 2009). Although there is no literature regarding the effect of VEGF on *HAS* expression, it is another growth factor that is present in the tumour microenvironment. VEGFs act on three receptors (VEGFRs) with tyrosine kinase activity (Koch S, 2012). In general VEGFR1 is expressed in monocytes and macrophages, VEGFR2 is expressed in vascular endothelial cells and VEGFR3 is expressed in lymphatic endothelial cells. The VEGFs are responsible for regulating endothelial cell migration, proliferation and consequent lymphangiogenesis through activation of pathways such as RAS/RAF/ERK/MAPK and PI3K/PDK/AKT (Koch S, 2012). VEGF-C has been shown to promote lymphangiogenesis in tumours and subsequent dissemination and metastasis in lymph nodes (Mandriota, 2001).

There is little information in the literature describing VEGF signalling in tumour progression. However, recent work has demonstrated that VEGF can increase motility in oral SCC cells *in vitro* via phosphorylation of the Akt pathway (Islam, et al., 2014).

Chapter 2 Materials and Methods

Cell Culture

MM1 cells are gingival fibroblasts provided by Dr M. Macluskey of Dundee Dental Hospital. COMD11, COMD24 and COMD25 cells are tumour-associated fibroblasts from oral squamous cell carcinoma (SCC) biopsies, donated by the Oral Surgery Clinic, Ninewells Hospital, Dundee, and isolated from explant cultures in Dundee Dental School. HaCaT cells are commercially available human spontaneously immortalised epidermal keratinocytes (Life Technologies), donated by Prof S Schor. These are used as a control cell line for the SCC cell lines, PM1, TYS and TR146. PM1 cells are taken from a dysplastic lesion from the forehead skin, donated by Dr D Crouch, Dundee Dental School. TR146 are a commercially available cell line taken from the lymph node of a patient with a well-differentiated SCC of the oral mucosa (Sigma Aldrich). TYS are taken from an oral adenoid SCC, derived from a minor salivary gland and donated by Dr K Harada, University of Tokushima, Japan. All samples were collected after receipt of ethical approval.

Cells to be stained were grown on 60 mm dishes in 10% (v/v) Foetal Calf Serum medium (FCS-MEM; see Appendix 1 for recipe) supplemented with 1% (v/v) glutamine until they reached their maximum confluency. However, not all cell lines became 100% confluent before detaching from the plate, so in these cases, their maximum confluency was approximated. They were then incubated with growth factors for 24 hours. TGFα (10-1033-B, Insight Biotechnology) or $VEGF_{121}$ (10-1296-B, Insight Biotechnology) were added at concentrations of 1pg/ml, 10 pg/ml, 100 pg/ml and 1ng/ml, whereas EGF (10-1001-B, Insight Biotechnology) was added at concentrations of 10pg/ml, 100pg/ml, 1ng/ml and 10ng/ml. HA (HA2002, Fermentech) was added at concentrations from 10ng to 10µg. Samples were then prepared as appropriate to their use.

Immunocytochemistry (ICC)

The cells were fixed in methanol for 20 minutes, and incubated in 1.0% (v/v) Triton x100 detergent in PBS for 5 minutes before beginning the ICC protocol. Firstly, the cells were incubated in 5% (v/v) Normal Goat Serum (NGS) in PBS-T for 30-60 minutes. The cells were then incubated with the primary antibody (see Appendix 2) overnight in a humidified chamber at 4˚C. It was found that staining worked best at a dilution of 1:50 (v/v) *HAS2* antibody in 5% NGS in PBS-T. Next the cells were incubated with the secondary antibody for 60-120 minutes. The secondary antibody was an anti-rabbit IgG conjugated with a fluorescent dye at 1:1000 (v/v) in 5% NGS in PBS-T. The fluorescent dye could then be detected with a UV fluorescence microscope with a light source and an inverted phase contrast microscope (Olympus IX70). Images were captured with Metamorph software and a 10 second exposure.

Statistical Analysis of ICC

Fluorescence intensity was calculated using ImageJ software (Research Services Branch, National Institute of Health). Due to the varied cell confluency and frequency between plates, five cells were selected in each photo and their fluorescence intensity recorded. The selection favoured cells that were free from overlap and artefact so that the final average taken would represent an average fluorescence per cell. Only five cells were selected as some fibroblast samples had only four or five cells in a photo. Three samples of background fluorescence were taken in each photo to determine an average to subtract from the cell data. The formula used to give the Corrected Total Cell Fluorescence (CTCF) can be found in Appendix 3.

The CTCF of each sample was normalised within each experiment (constant cell type, varied growth factor concentration) by dividing by the mean of all the CTCFs in the experiment. A Pearson's Correlation Coefficient was determined for each experiment to identify correlations. With a P value of 0.05, the critical value for r = 0.878. However, where r > 0.5, a correlation may be considered reasonably strong although it is not statistically significant.

HA ELISA Assay

Cells were incubated with the growth factors EGF or VEGF for 24 or 48 hours. The collection media were then centrifuged and decanted to remove any cells and frozen.The DuoSet Assay Development kit for hyaluronan from R and D Systems (Cat No DY3614) was used according to manufacturer's instructions.

96-well plates were used and incubated with capture reagent overnight (all materials can be found in Appendix 4). The plates were blocked and filled with the samples, diluted in reagent diluent. In order to create a standard curve of optical densities against HA concentration, a HA standard was diluted to concentrations from 30ng/ml to 0.123ng/ml and included in every plate. The samples were incubated at room temperature for 2 hours. The plates were subsequently incubated with the detection reagent for 2 hours, then Streptavidin-HRP (1:2000) for 20 minutes, the substrate solution for 20 minutes and finally the stop solution was added. The optical densities of the samples were then read at 450nm and 570 nm and analysed using the FLUOstar OPTIMA (BMG Labtech) system. The results were then exported to Microsoft Excel for analysis and creation of a standard curve.

SDS PAGE and Western Blot

Samples were prepared from cells lysed *in situ* with RIPA buffer and Protease Inhibitors (Ref 11836170001 Roche). Samples contained equal volumes of cell lysate and Laemmli loading buffer with 5% v/v 2-mercaptoethanol (#161-0737 Bio-Rad), and were heated at 95˚C for 5 minutes before being loaded onto a 10% BioRad TGX Gel. Gels also contained a sample of Magicmark XP molecular weight markers (LC5603 Invitrogen).

The gels were run according to the manufacturer's instructions with TGS running buffer (See Appendix 2 for all buffer recipes) then blotted (semi-dry 15V 42 minutes) onto nitrocellulose membranes using Towbin transfer buffer (68) and blocked using 1% (w/v) fat free milk TBST. They were then incubated with primary rabbit antibodies for vimentin (#3932 Cell Signaling; diluted 1:2000 in 1% (w/v) Milk TBST) and E-Cadherin (#3195S Cell Signaling; diluted 1:1000 in 1% (w/v) Milk TBST) overnight with orbital shaking. The following day, the blots were washed with TBST three times for 20 minutes with orbital shaking and incubated with a goat anti-rabbit secondary antibody (#7074 Cell Signaling) diluted 1:2,000 in 1% (w/v) milk TBST, for an hour. The TBST washes were then repeated before adding 0.7ml of Immun-Star Western C substrate (BioRad), which displays chemiluminescence in the presence of Horse Radish Peroxidase (HRP), attached to the secondary antibody. This was detected by a Biorad ChemiDoc MP Imaging System. Imagelab software was used to analyse the visualised blots.

Scratch Assay

The cells were cultured until confluent and incubated with SF-MEM for 24 hours before the scratch was carried out. A flexible pipette tip was used to 'scratch' a line down the monolayer of cells without scoring the plastic of the dish. Pictures were taken at this point

as a starting time point (t^0). The cells were then incubated with HA at concentrations from 10ng to 10 µg with a SF-MEM negative control. The dishes were checked at 3 hours, 6 hours and 24 hours for changes and further photos were taken if changes had occurred.

Boyden Chamber Assay

The cell samples were passaged the day before harvesting and on the day of harvesting, as it has been shown in our lab that confluent cells are less suitable for this assay. This assay follows the technique used by Schor *et al.,* 1996, 1999, 2006.

Polyvinylpropylene-free polycarbonate Nucleopore membranes (Fisher 8.0µm pore) were coated with native type I collagen by overnight immersion in a 100µg/ml aqueous solution at 4°C and air dried before being fitted into a 48-well microchemotaxis chamber (Neuroprobe Inc). The lower wells contained the indicated peptide fragment in serum free (SF-MEM) plus 2µg/ml bovine serum albumin (BSA) in assays designed to measure chemotaxis. A total of $12.5x10^4$ cells suspended in 50µl SF-MEM + 2µg/ml BSA were then plated into the upper wells. The chambers were incubated for 5-6 hours at 37°C in a humidified CO_2 incubator and the membranes then removed, fixed in methanol and stained with Mayers Haemotoxylin. Cells remaining on the top of the membrane were removed, the membranes mounted onto glass slides and examined under bright field illumination at a magnification of 200x. The number of migrated cells adherent to the lower surface of the membrane were counted in three fields per well and 6 wells per variable (i.e. 18 fields per variable). Data are expressed as mean cell number and standard error per field.

Chapter 3 Results

HAS2 and HAS3 Levels in Fibroblast and Epithelial Cells by Immunocytochemistry

This experiment used three epithelial cell lines from Squamous Cell Carcinomas (PM1, TR146, TYS) and one normal epithelial control (HACAT), as well as three tumour-associated fibroblast lines (COMD11, COMD24, COMD25) and one normal fibroblast control (MM1). The cell lines were grown until at least 80% confluency before a 24 hour incubation with either EGF, TGFβ1 or VEGF over a range of concentrations. The cells were then fixed in methanol and an immunohistochemical (ICC) staining procedure was used for *HAS2* and *HAS3*. The secondary antibody was linked to an Alexa Fluor dye for detection with fluorescence microscopy. In this way, *HAS2* and *HAS3* localisation was viewed as well as quantified by analysis of fluorescence intensity using ImageJ.

The data were normalised separately within their experimental runs, giving two sets of data ("Normalised 1" and "Normalised 2"). From these data, the Pearson's Correlation Coefficient (r) was calculated to determine linear correlations. These r values are given in Table 7.

HAS2 and *HAS3* Distribution and Localisation

Of the HAS isoforms, HAS1 is usually found in adults whereas HAS2 and HAS3 are found during inflammation or in embryological development. However, in the cell lines tested, the presence of HAS2 and HAS3 was almost ubiquitous. In the majority of samples, 100% of cells stained for HAS2 and HAS3.

ICC showed HAS2 and HAS3 were present evenly throughout the cells, which is to be expected since it is a plasma membrane protein. In some fibroblast photos, it seems as if the stain is more concentrated over the nucleus (See Figure 8). However, this is most likely due to the elongated fibroblast shape with a thick cell body surrounded by thinner extensions of cytoplasm.

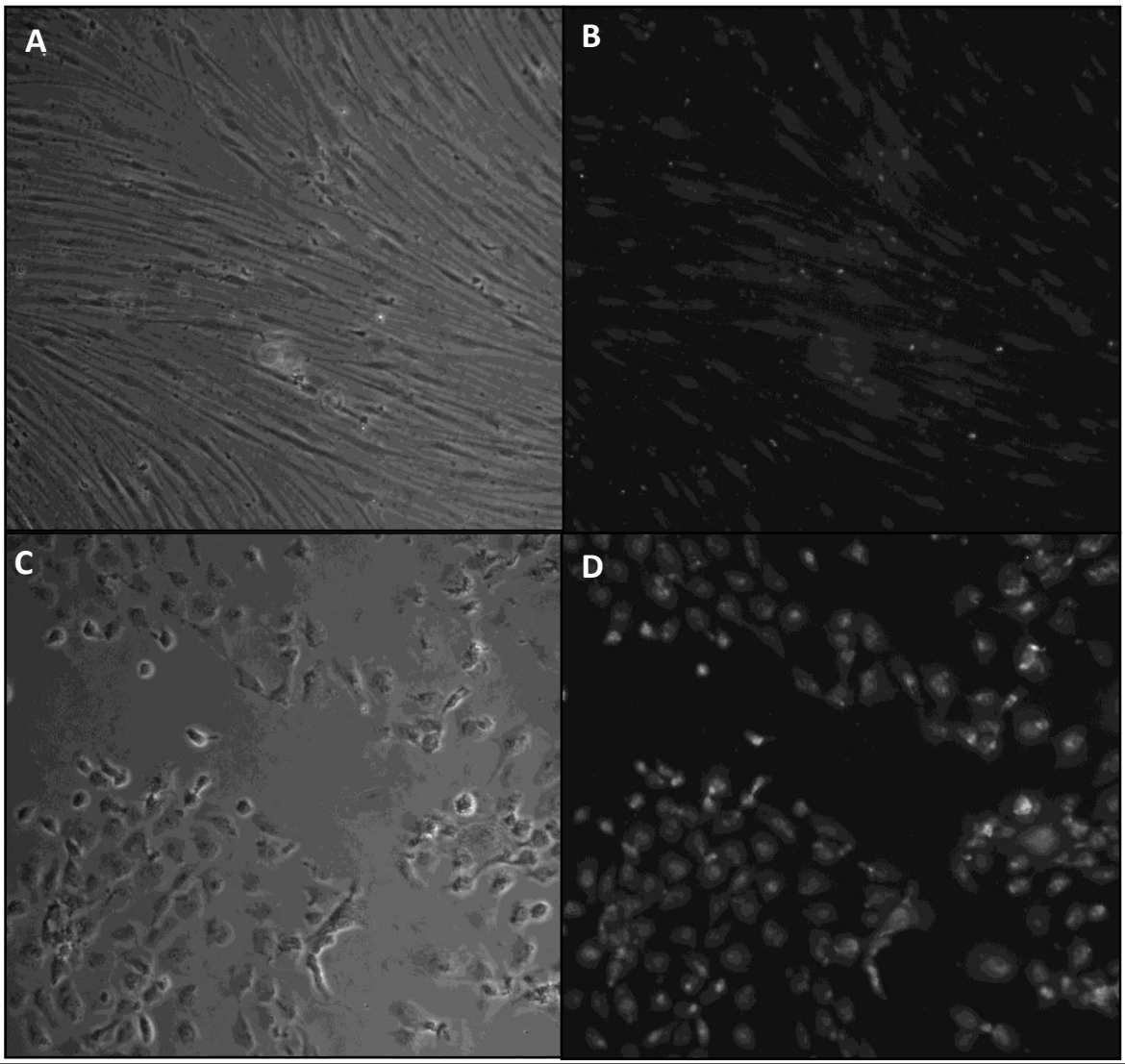


Figure 8 Images of HAS2 distribution in fibroblast and epithelial cells. A) Phase contrast microscopy of MM1 incubated with 100pg VEGF. B) Fluorescence microscopy of A after incubation with anti-HAS2 primary antibody and fluorescent secondary antibody. C) Phase contrast microscopy of TR146 incubated with 10ng VEGF. D) Fluorescence microscopy of C after incubation with anti-HAS2 primary antibody and fluorescent secondary antibody. Note that all cells stained for HAS2 throughout the whole cell surface.

Cell Line	EGF		TGFβ1		VEGF	
	HAS2	***HAS3***	***HAS2***	***HAS3***	***HAS2***	***HAS3***
HACAT	0.757	0.826	-0.135	-0.119	0.333	-0.559
PM1	0.363	-0.802	-0.164	-0.484	-0.597	-0.144
TR146	0.977	0.959	0.595	0.035	-0.032	-0.124
TYS	-0.214	-0.612	0.874	-0.035	-0.310	-0.562
MM1	0.954	0.694	-0.143	-0.341	-0.801	-0.314
COMD11	0.113	0.825	-0.443	0.079	0.353	0.947
COMD24	-0.863	-0.197	-0.312	-0.456	0.725	0.051
COMD25	0.897	0.518	0.256	0.177	-0.556	0.096

Table 7. Pearson's Correlation Coefficient values between the concentration of growth factor added and the fluorescence intensity of *HAS2* or *HAS3* stain, calculated using ImageJ software; p=0.05 highlighted in dark green; p=0.1 highlighted in grey.

Effects of EGF

The relative HAS2 and HAS3 levels in epithelial cells and fibroblasts grown under varied EGF levels are shown in Figures 9-12. The literature would suggest EGF promotes HAS2 and HAS3 production in some epithelial and fibroblast lines.

The data show a strong correlation (r=0.757) between EGF and HAS2 in the HACAT control line and a significant positive correlation (r=0.977) in TR146, which is consistent with the literature. However, this relationship is somewhat disturbed in PM1 and TYS cells. A similar situation is found with HAS3; HACAT and TR146 show strong positive correlations (r=0.826 and r=0.959 respectively) while strong negative correlations are found in the other two cell lines.

Regarding the fibroblast cell lines, a significant positive correlation (r=0.954) is found between EGF and HAS2 in the control MM1 line and in COMD25 cells. This is lost in COMD11 and COMD24. The correlation with HAS3 levels is not as strong in the MM1 cells (r=0.694) but is nonetheless disturbed in COMD cell lines.

Effects of TGFβ1

Figures 13-16 show the relative HAS2 and HAS3 levels in epithelial cells and fibroblasts grown under varied TGFβ1levels. The literature suggests that TGFβ1decreases HAS2 and HAS3 production in some epithelial and fibroblast lines.

The data here show no strong correlations of HAS2 or HAS3 with TGFβ1in the control HACAT cells. However, positive correlations occur in TR146 and TYS HAS2 levels (r=0.595 and r=0.874 respectively). This is not the expected relationship but may be another case of disturbed cell signalling pathways in transformed cells.

The control fibroblast line MM1 seems to undergo an increase of HAS2 and HAS3 at lower concentrations of TGFβ1followed by a decrease at higher concentrations. This relationship is conserved in some COMD cell lines and not in others. This is another case where signalling seems to be altered in tumour-associated cells.

Effects of VEGF

Figures 17-20 show the relative HAS2 and HAS3 levels in epithelial cells and fibroblasts grown under varied VEGF concentrations. The effects of VEGF on HAS expression have not been as well documented as those of EGF and TGFβ1. On increasing VEGF concentrations in epithelial cell cultures, HAS2 and HAS3 appeared to increase at low concentrations of VEGF and decrease at higher concentrations.

VEGF showed a strong negative correlation (r=-0.801) with MM1 HAS2 levels, which was fairly conserved in the COMD cell lines. HAS3 increased at low VEGF concentrations and decreased at high VEGF concentrations in MM1 cells. However, this too was less pronounced, if not absent, in COMD cells.

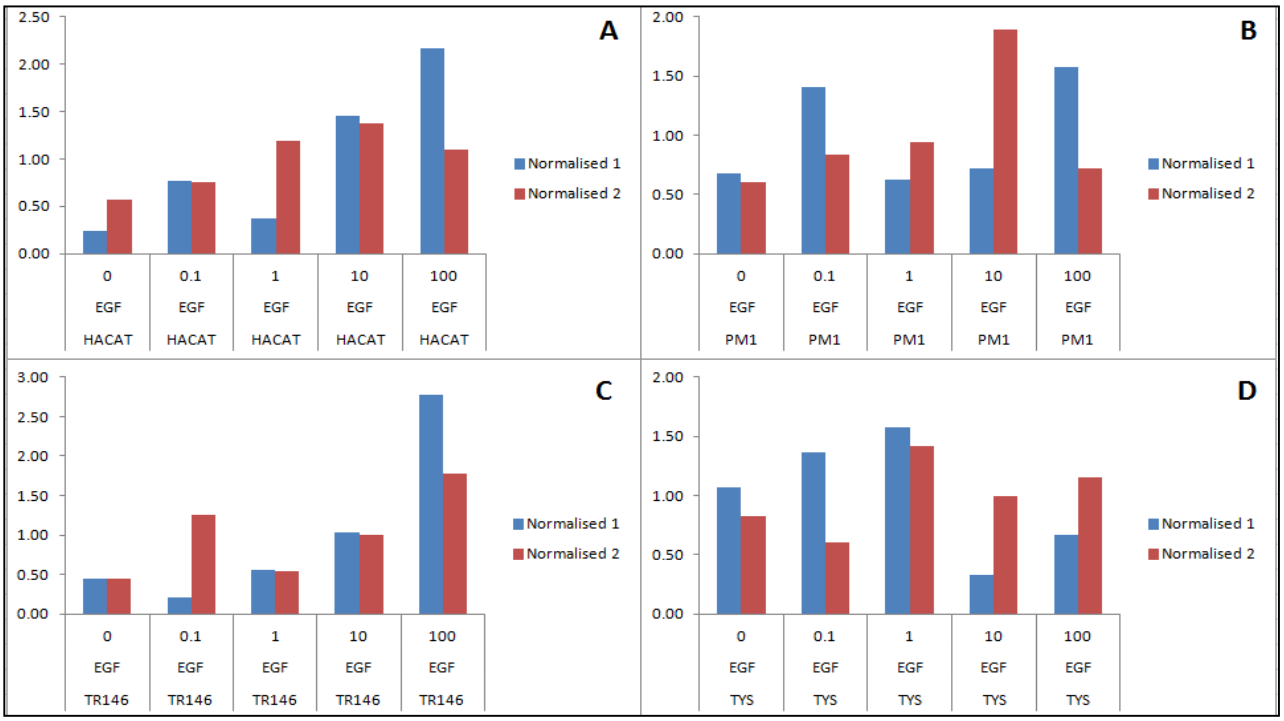


Figure 9 HAS2 levels expressed in epithelial cells treated with different concentrations of EGF, measured by fluorescence intensity and run in duplicate. Please note that the data have been normalised separately within their experimental runs and are not relative to each other. A) HACAT cells B) PM1 cells C) TR146 cells D) TYS cells. There are positive correlations between EGF and HAS2 in the HACAT control line (A) and TR146 (C), which is consistent with the literature. This relationship is less in PM1 and TYS cells (B and D).

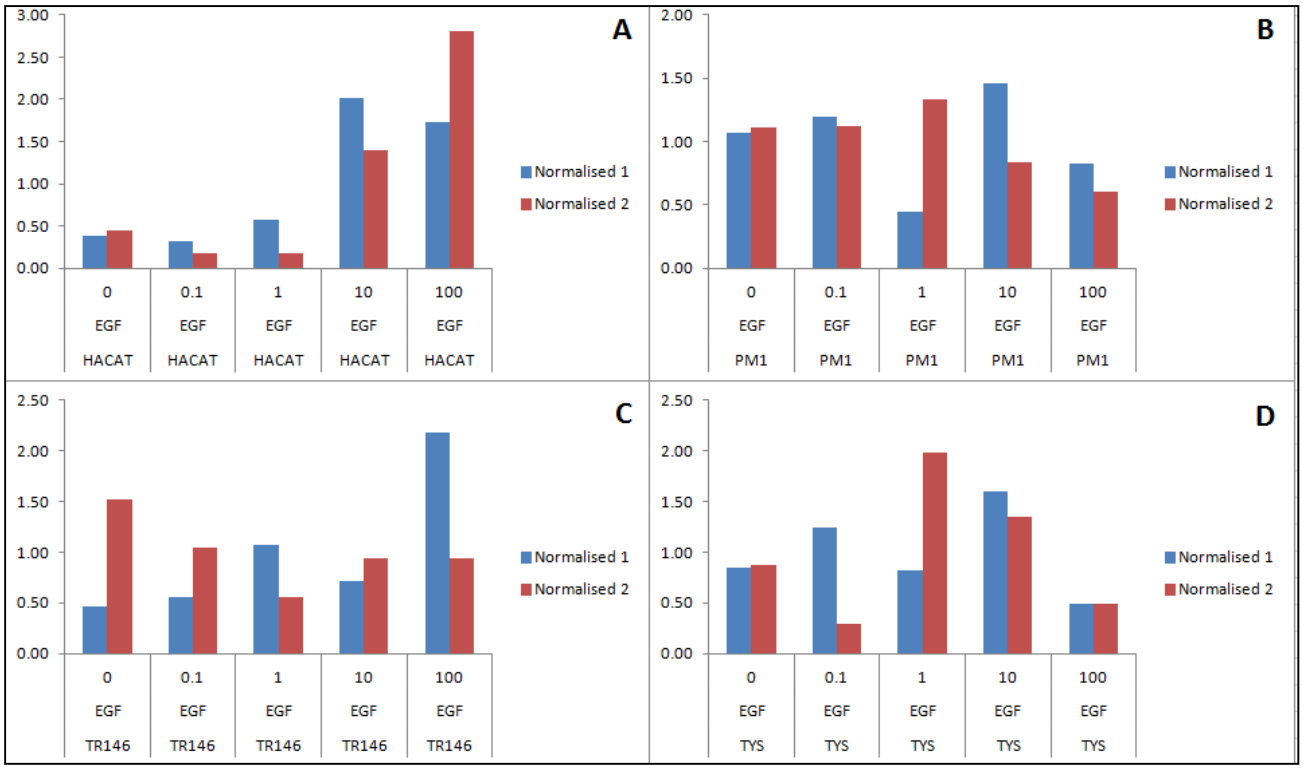


Figure 10 HAS3 levels expressed in epithelial cells treated with different concentrations of EGF, measured by fluorescence intensity and run in duplicate. Please note that the data have been normalised separately within their experimental runs and are not relative to each other. A) HACAT cells B) PM1 cells C) TR146 cells D) TYS cells. HACAT and TR146 show positive correlations between increasing EGF and HAS3 while PM1 and TYS show negative correlations.

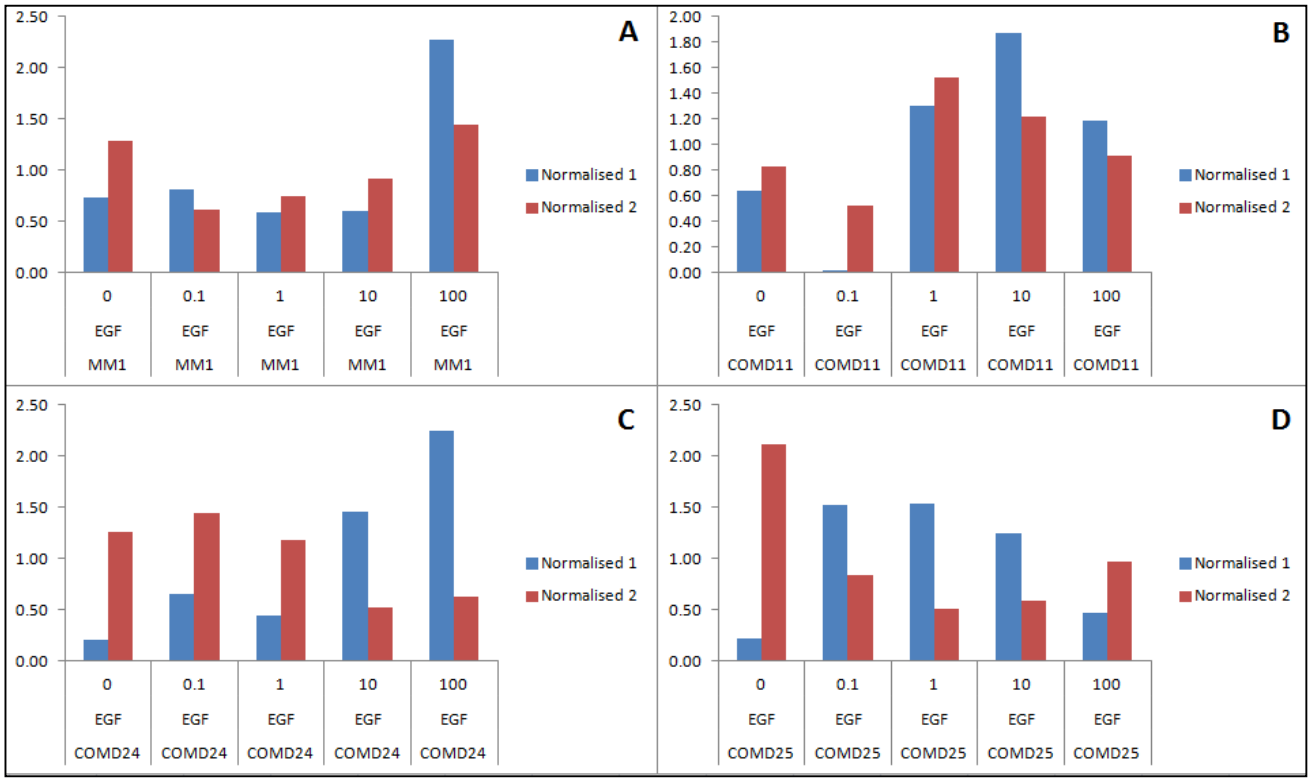


Figure 11 Relative HAS2 levels expressed in fibroblast cells treated with different concentrations of EGF, measured by fluorescence intensity and run in duplicate. A) MM1 cells B) COMD11 cells C) COMD24 cells D) COMD25 cells. There is a significant positive correlation between EGF and HAS2 in the control MM1 line (A) and in COMD11 cells (B). There is no correlation in COMD24 (C) and a negative correlation in COMD25 (D).

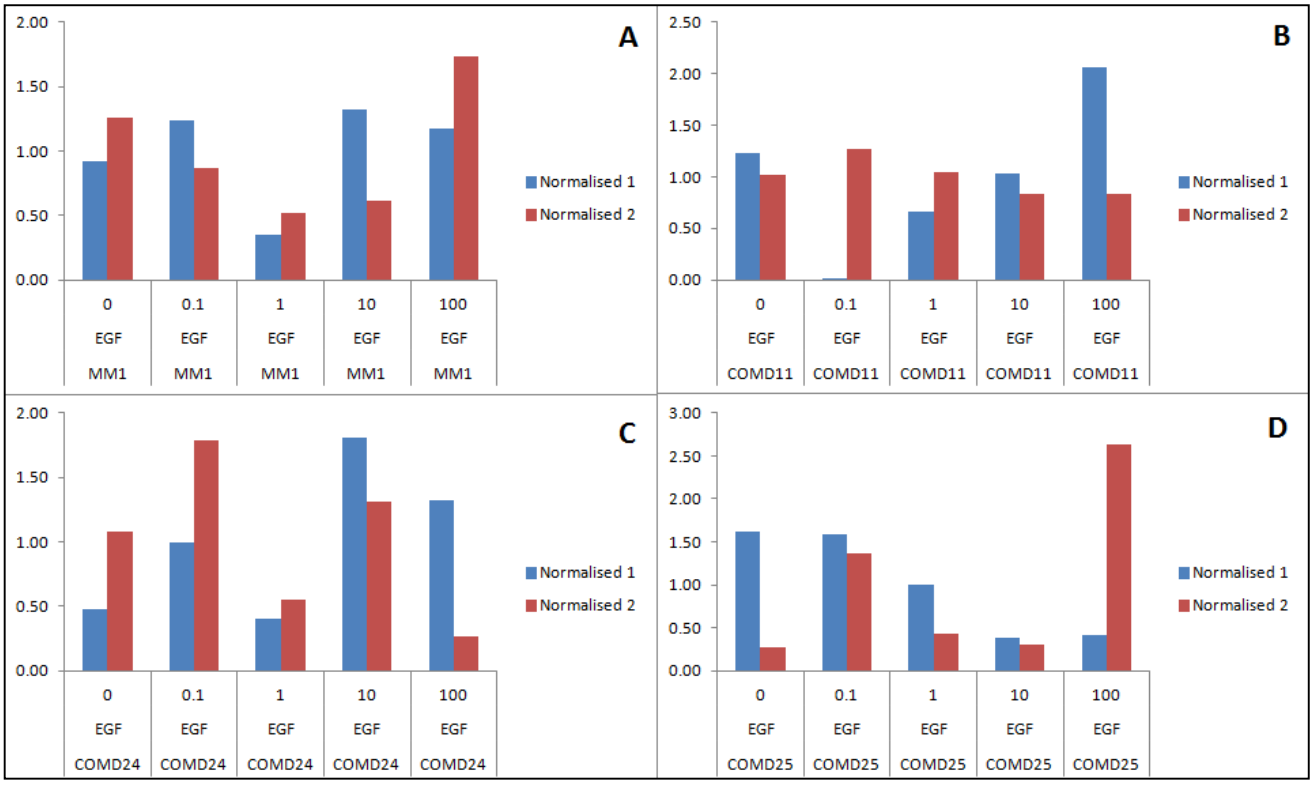


Figure 12 Relative HAS3 levels expressed in fibroblast cells treated with different concentrations of EGF, measured by fluorescence intensity and run in duplicate. A) MM1 cells B) COMD11 cells C) COMD24 cells D) COMD25 cells. The relationship between EGF and HAS3 levels is not consistent. A differential effect at low levels and high levels of EGF can be seen in MM1 (A) and COMD11 (B).

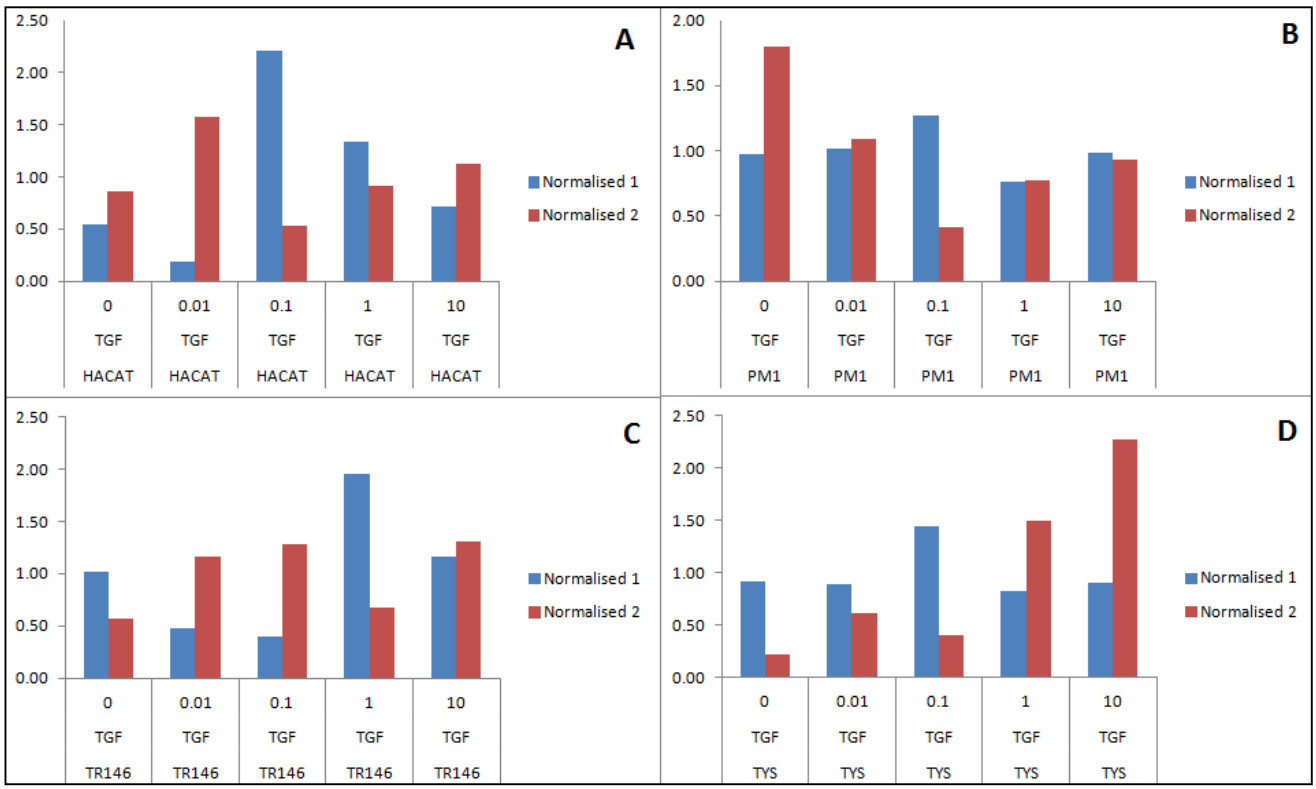


Figure 13 Relative HAS2 levels expressed in epithelial cells treated with different concentrations of TGFβ1, measured by fluorescence intensity and run in duplicate. A) HACAT cells B) PM1 cells C) TR146 cells D) TYS cells. There is no correlation evident in the HACAT cell line between TGFβ1 and HAS2. There are positive correlations in TR146 and TYS HAS2. This differs from the expected relationship reported in the literature (Pasonen-Seppänen., 2003).

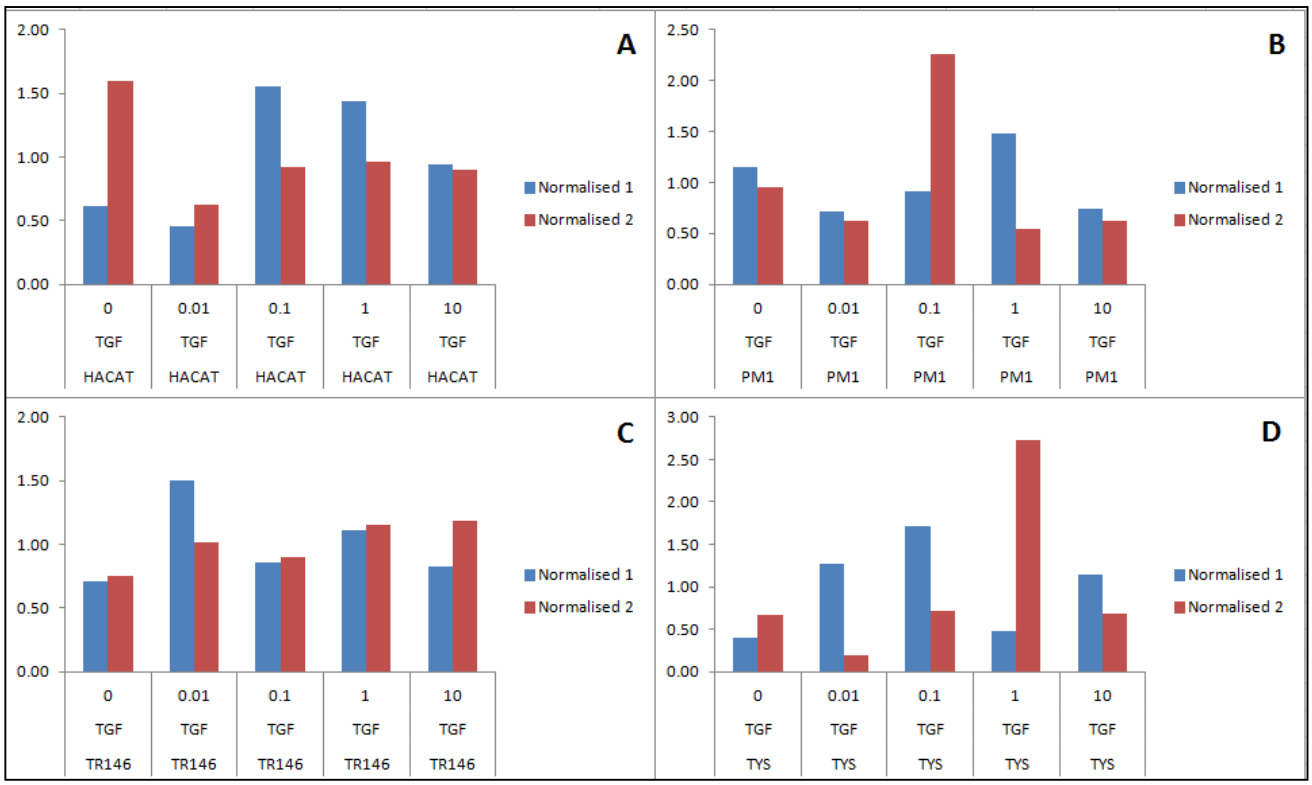


Figure 14 Relative HAS3 levels expressed in epithelial cells treated with different concentrations of TGFβ1, measured by fluorescence intensity and run in duplicate. A) HACAT cells B) PM1 cells C) TR146 cells D) TYS cells. There is a positive correlation between TGFβ1 and HAS3 in the HACAT and TR146 cell lines. This is less consistent in PM1 and TYS.

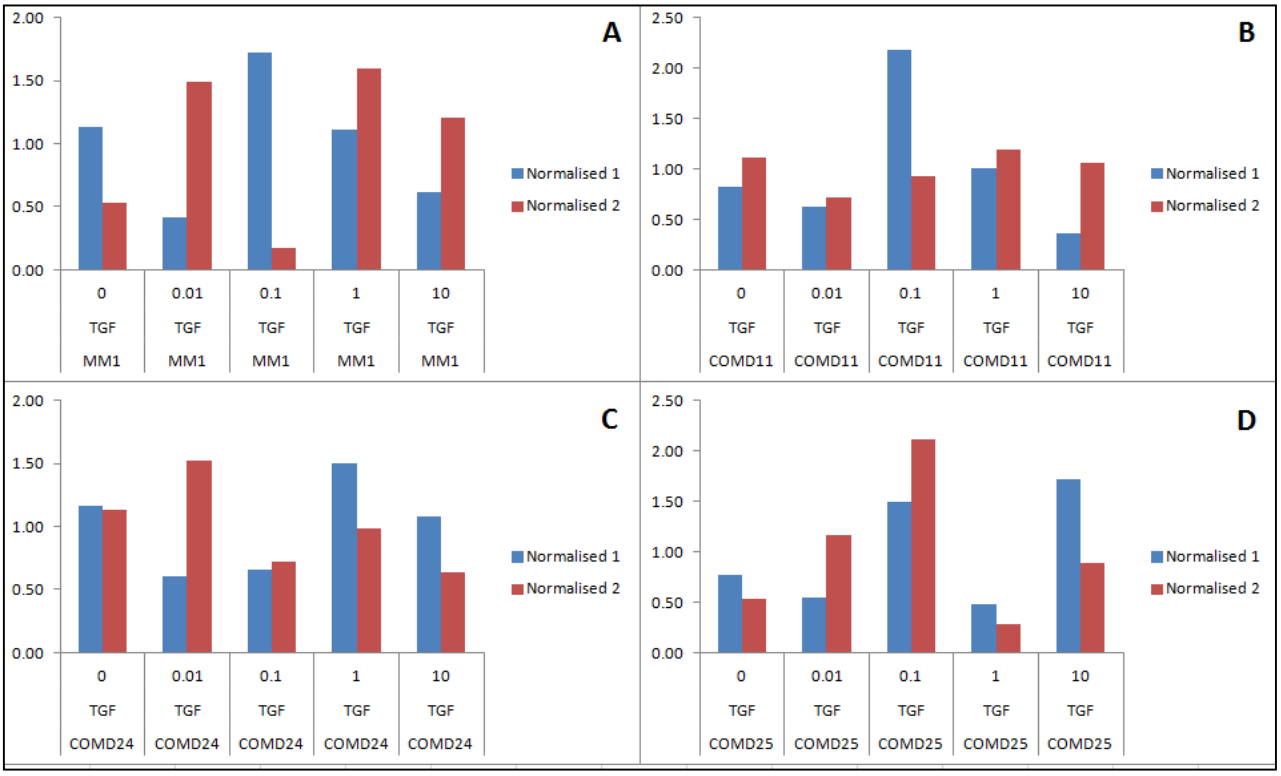


Figure 15 Relative HAS2 levels expressed in fibroblast cells treated with different concentrations of TGFβ1, measured by fluorescence intensity and run in duplicate. A) MM1 cells B) COMD11 cells C) COMD24 cells D) COMD25 cells. There is generally an increase in HAS2 at low concentrations of TGFβ1 and less of an effect or even a decrease in HAS2 at higher concentrations of TGFβ1.

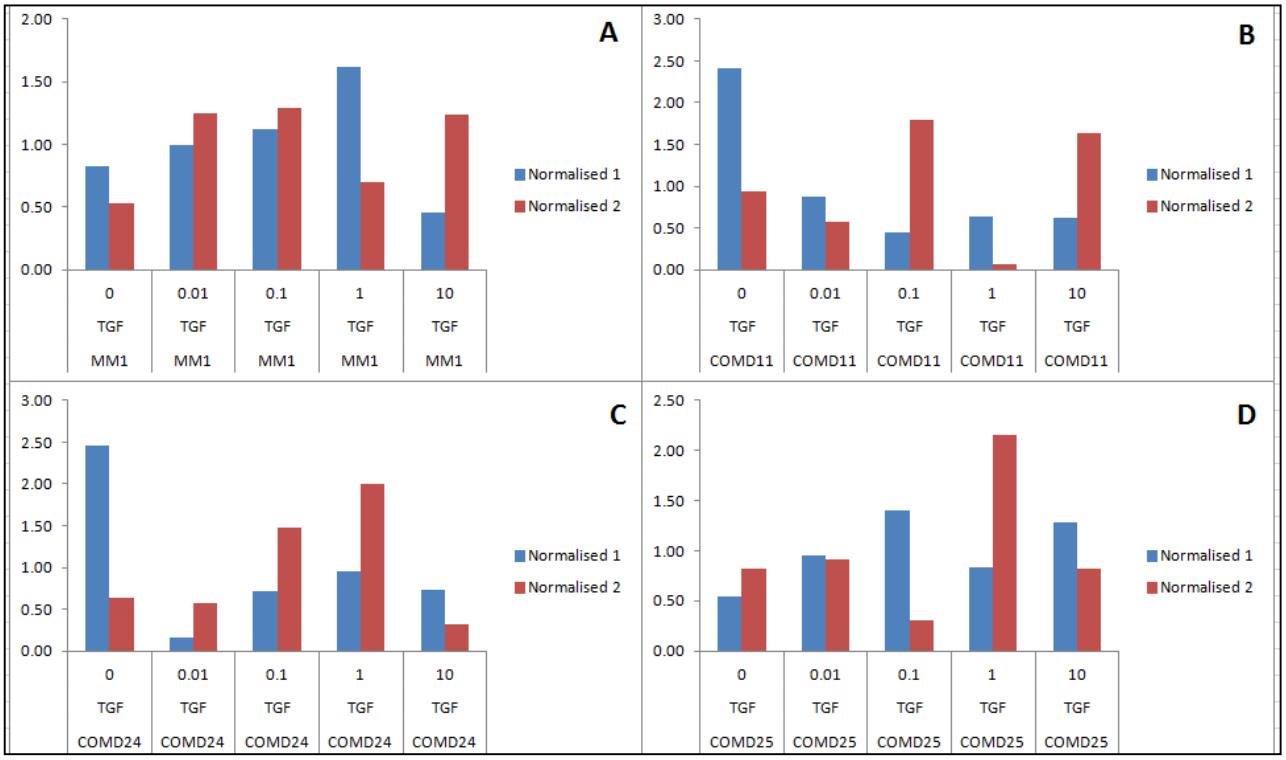


Figure 16 Relative HAS3 levels expressed in fibroblast cells treated with different concentrations of TGFβ1, measured by fluorescence intensity and run in duplicate. A) MM1 cells B) COMD11 cells C) COMD24 cells D) COMD25 cells. In MM1 cells, there is an increase of HAS3 at lower concentrations of TGFβ1 and a decrease at higher concentrations. However, this relationship is not consistent in COMD cell lines

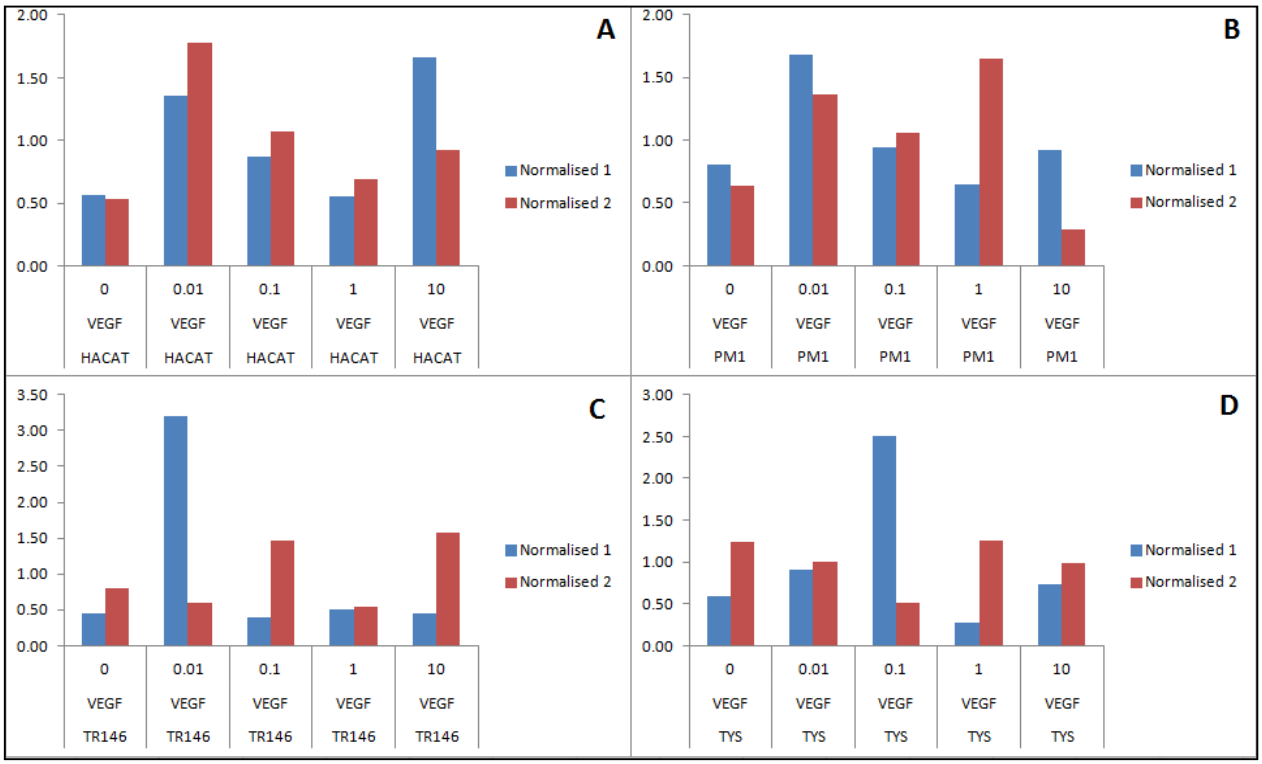


Figure 17 Relative HAS2 levels expressed in epithelial cells treated with different concentrations of VEGF, measured by fluorescence intensity and run in duplicate. A) HACAT cells B) PM1 cells C) TR146 cells D) TYS cells. The general trend is an increase of HAS2 at low concentrations of VEGF and less effect or even a decrease of HAS2 at higher concentrations.

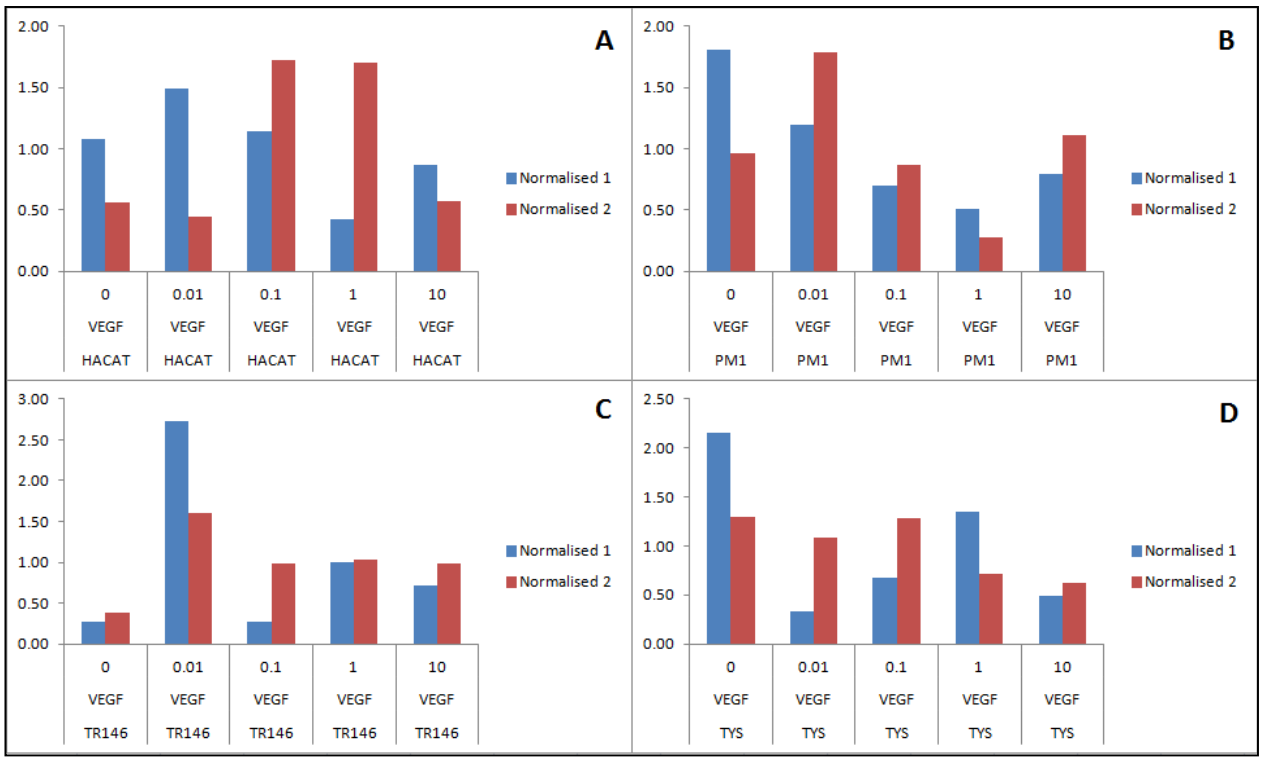


Figure 18 Relative HAS3 levels expressed in epithelial cells treated with different concentrations of VEGF, measured by fluorescence intensity and run in duplicate. A) HACAT cells B) PM1 cells C) TR146 cells D) TYS cells. There appears to be an increase of HAS3 at lower concentrations and a decrease at higher concentrations, similar to the relationship seen with HAS2. However, the TYS cell line appears to only show a decrease in HAS3 levels.

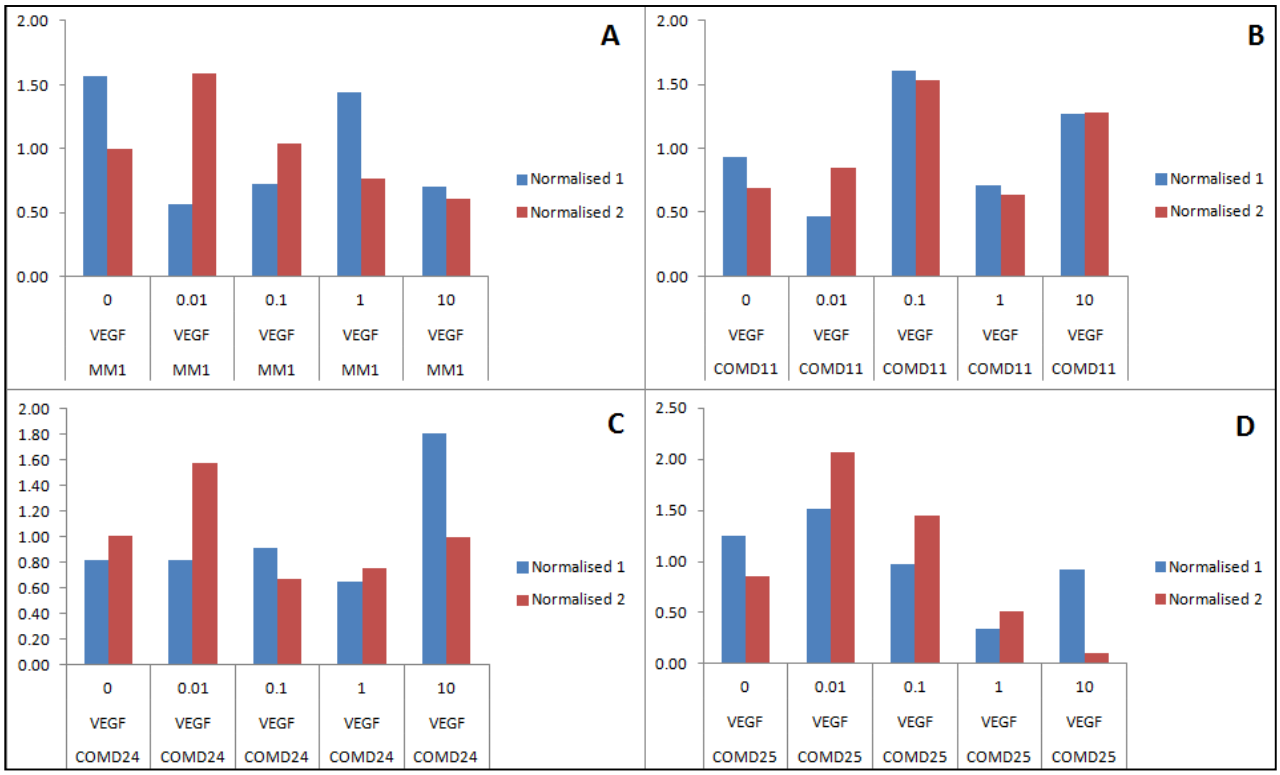


Figure 19 Relative HAS2 levels expressed in fibroblast cells treated with different concentrations of VEGF, measured by fluorescence intensity and run in duplicate. A) MM1 cells B) COMD11 cells C) COMD24 cells D) COMD25 cells. VEGF showed a strong negative correlation with HAS2 levels in MM1. This was conserved in COMD25 but lost in the other COMD cell lines.

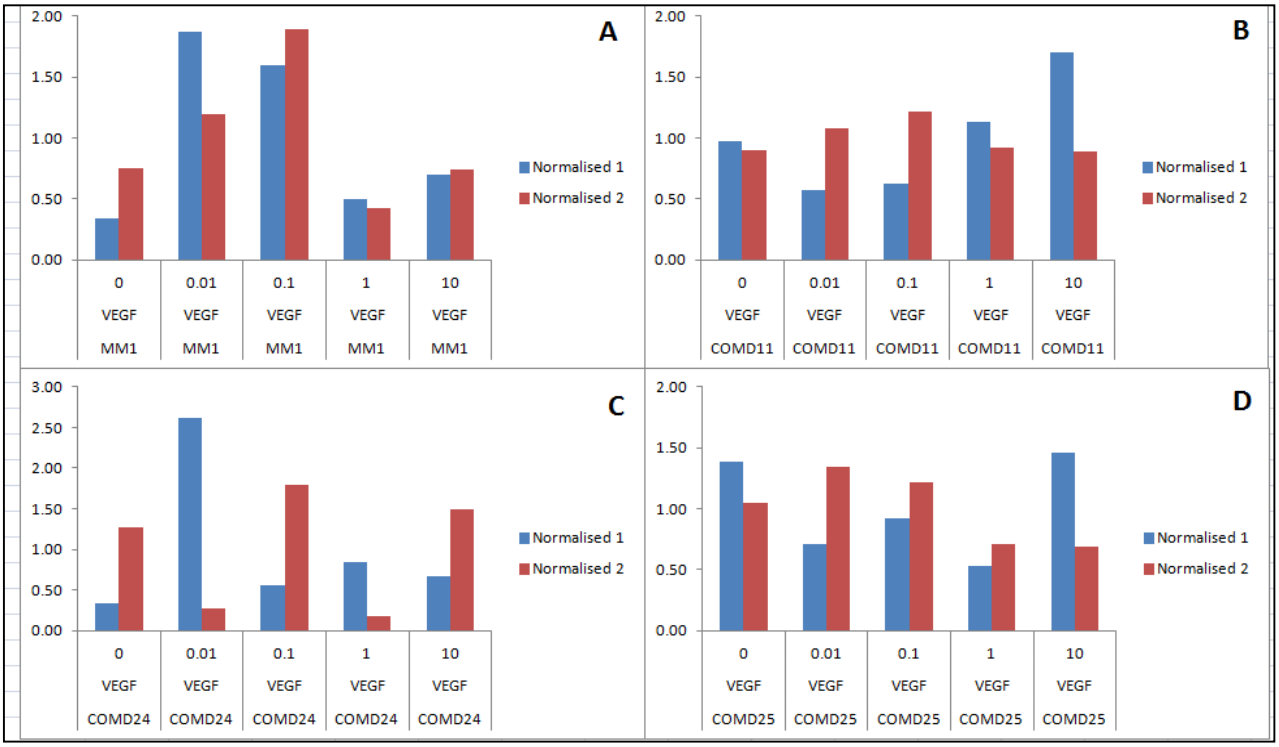


Figure 20 Relative HAS3 levels expressed in fibroblast cells treated with different concentrations of VEGF, measured by fluorescence intensity and run in duplicate. A) MM1 cells B) COMD11 cells C) COMD24 cells D) COMD25 cells. HAS3 increased at low VEGF concentrations and decreased at high VEGF concentrations in MM1 cells. This relationship was lost in COMD cell lines.

Summary of Growth Factor Effects

These experiments have looked at the effects of growth factors individually, and the results frequently differ between tumour-associated cell lines and the epidermal control line, suggesting that signalling is altered or disturbed in tumour-associated cells. However, in vivo all these growth factors would be present simultaneously. When the data is put together it would seem that HAS2 synthesis is upregulated in epithelial cells. The positive effect of EGF may be conserved, while an inductive effect by TGFβ1 may be gained. The effect of VEGF depends upon the levels found in the tumour. HAS3 levels in epithelial cells are subject to conflicting effects from different growth factors. It may be that HAS3 has a lesser role in cell signalling between transformed epithelial cells.

No consistent patterns were seen in fibroblast HAS2 and HAS3 levels. The relationship seen in the MM1 control was usually changed by the growth factor but not in any predictable way.

In summary, both fibroblasts and TR146 cells experience an increase in HAS2 expression (and sometimes HAS3) on addition of EGF. Conversely, TYS increased HAS2 expression on addition of TGFβ1. Squamous Cell Carcinomas (SCC) undergo Epithelial to Mesenchymal Transition (EMT) as they become more advanced. This may be responsible for the varied responses seen in different epithelial tumour cell lines. Alternatively, this may be a case of convergent mutations, both leading to increased HA output.

Quantitative HA Assay in Epithelial and Fibroblast Cells

In order to determine whether changes in HA levels are proportional to changes in HAS levels, this experiment used the same epithelial cell (HACAT, PM1, TR146, TYS) and three

fibroblast cell lines (MM1, COMD25/11). Since the trends found in the ICC experiments were not always linear, two concentrations of EGF and VEGF were chosen to represent even non-linear relationships. The epithelial cell lines were grown until at least 80% confluency before a 24 hour incubation with either EGF or VEGF. Two samples of confluent fibroblast cell lines were incubated with EGF and VEGF for 24 hours and 48 hours respectively. The conditioned medium was taken and spun down to remove any cells. An indirect sandwich hyaluronan assay was used to quantify short chain HA levels by measuring optical density (OD) at 450nm and correcting these measurements against the background OD. Standard curves were produced in each assay, an example of which is shown in Figure 21. The results shown in Figures 22-23 show the HA quantities, calculated from the standard curves.

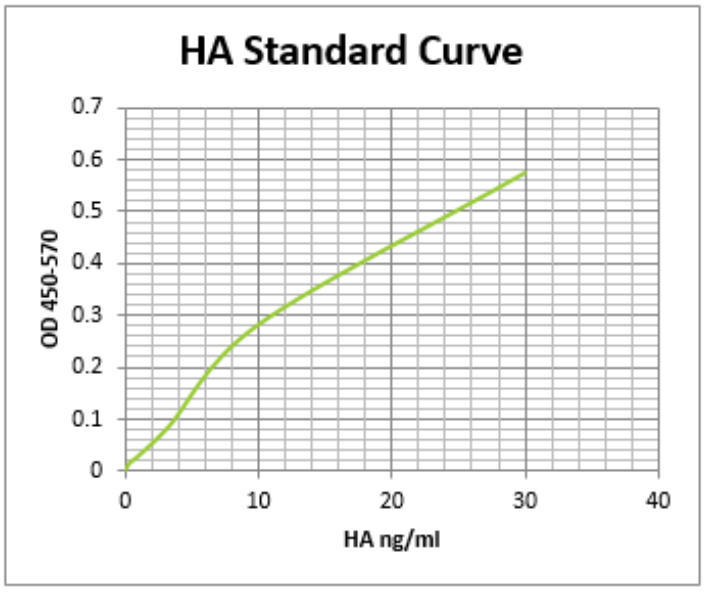


Figure 21 Example HA standard curve calculated from set HA concentrations, measured in the same assay as the experimental data. Note that each data set therefore used a different standard curve.

HA in Epithelial Cell Lines

All of the epithelial cell lines produced HA levels of more than 8.5ng/ml. However, the TYS and TR146 cell lines produced noticeably less than HaCaT. Interestingly, EGF and VEGF also gave different effects on HaCaT and PM1 lines, as opposed to TYS and TR146.

In TR146 lines, HA increased on addition of EGF, as predicted by the increase in *HAS2* levels, revealed by the fluorescent ICC. Conversely, the HaCaT, TYS and PM1 lines showed a decrease in HA levels despite a positive correlation between EGF and HAS2 levels. This could suggest another level of regulation affecting net HA production, such as catalytic activity of the enzyme, availability of substrate or increased degradation in the ECM.

VEGF had a less clear role, possibly causing an increase in *HAS2* and *HAS3* at low concentrations and a decrease at high concentrations. Corresponding HA levels showed little change in HaCaT cells, a decrease on addition of growth factors in PM1 cells, an increase in TR146 and a mixed result in TYS. This would again suggest additional levels of regulation.

HA in Fibroblast Cell Lines

All the fibroblast lines produced more than 26ng/ml HA. Generally, there was less HA in samples taken at 48 hours than 24 hours. The exception seen in Figure 23 was not present in the duplicate assay so it was not reproducible.

HA increased on addition of low concentrations of EGF in normal and tumour-associated fibroblasts. This corresponds to the increase in *HAS2* seen in the ICC experiments. HA also increased on addition of low levels of VEGF, which corresponds to the increase in *HAS3* seen in the ICC experiments. The trend in fibroblasts is that low levels of growth factors have larger effects than higher concentrations. This could be due to additional levels of regulation such as antisense RNA, which may be synthesised at high concentrations of HA. It is reported in the literature that TGFβ1 and EGF have optimum concentrations for eliciting peak *HAS* expression and peak migration (Grant, 1992). The optimum concentration of TGFβ1 is consistently lower than that of EGF, so it may be that the optimum concentration of VEGF is similarly low.

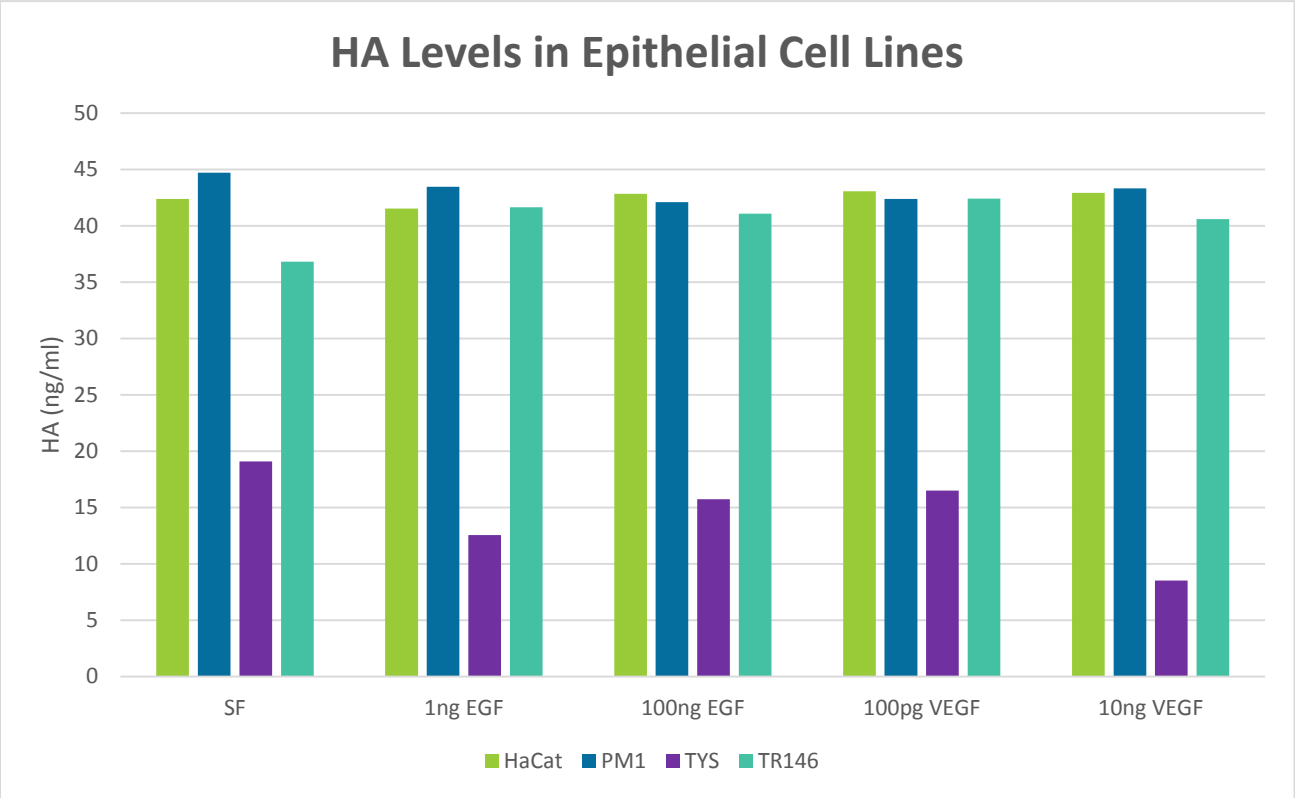


Figure 22 HA levels in epithelial cell lines as calculated from a standard curve derived from the same assay. All cell lines produced HA levels of more than 8.5ng/ml, although TYS showed lower levels than the control. HaCaT, PM1 and TYS lines showed a slight decrease in HA levels on addition of EGF and VEGF, whereas TR146 showed an increase in HA with both EGF and VEGF.

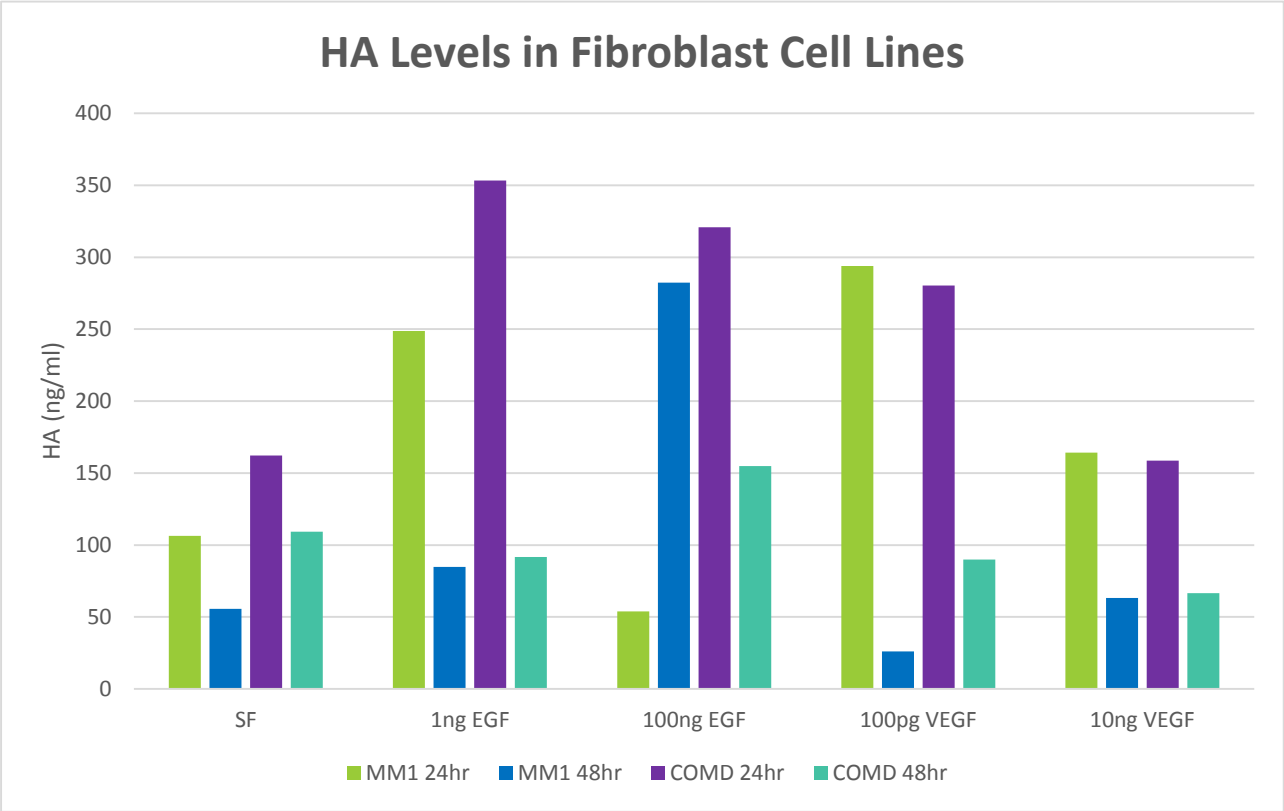


Figure 23 HA levels in fibroblast cell lines as calculated from a standard curve derived from the same assay. All the fibroblast lines produced more than 26ng/ml HA. HA levels were lower in samples taken at 48 hours compared to 24 hours. The exception seen in MM1 with 100ng EGF was not present in the duplicate assay. HA levels increased in both normal and tumour-associated fibroblasts, on addition of low concentrations of EGF and VEGF. At higher concentrations, the effect was less. Also, EGF gave a stronger response than VEGF.

EMT Markers – Vimentin and E-Cadherin

Having demonstrated changes in HA levels in the cultured stromal cells, this project will now attempt to determine its implications. It has been reported that HA levels are involved in Epithelial to Mesenchymal Transition (EMT), an important stage in tumorigenesis. In order to determine if EMT is taking place, the four epithelial cell lines were grown to confluency and incubated with decreasing concentrations of HA between 10µg and 10ng for 24 hours. The cells were then lysed and prepared for the SDS-PAGE protocol and subsequent Western blotting. The primary antibodies used were anti-vimentin and anti-E-cadherin. Vimentin is an intermediate filament present in mesenchymal cells whereas E-cadherin is an adhesive transmembrane protein present in epithelial cells.

The PM1 cell line is unusual in that it does not express any E-cadherin but does express large quantities of vimentin, even in the HA-negative control. This could suggest it is not epithelial in the first place.

E-Cadherin expression was unchanged by increasing HA concentration. However, vimentin expression was switched on in TR146 on increasing HA concentration, which could be an early sign of EMT.

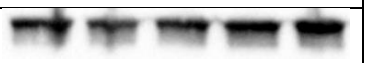
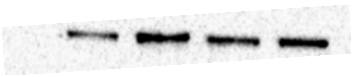
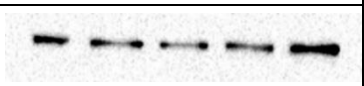
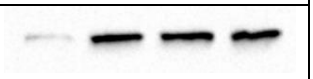
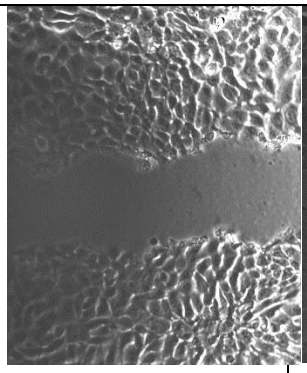
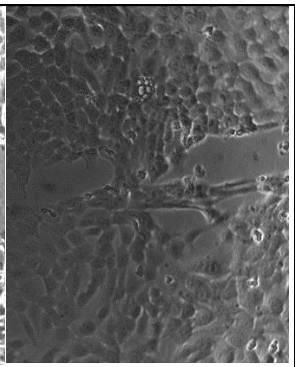
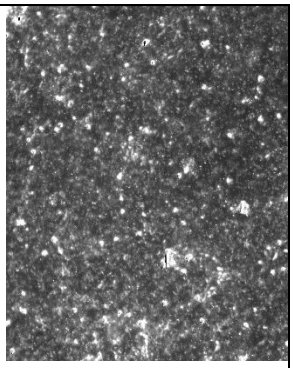
		E-Cadherin						Vimentin				
Concentration HA (µg/ml)	(kDa)	0	0.01	0.1	1	10	(kDa)	0	0.01	0.1	1	10
HaCaT	97 →						57 →					
PM1	97 →						57 →					
TYS	97 →						57 →					
TR146	97 →						57 →					

Table 8. Western blots detecting E-Cadherin and Vimentin. PM1 does not show an epithelial phenotype profile. HACAT and TYS show no change on addition of HA. TR146 does not show any change in E-Cadherin expression but gains vimentin expression, which could indicate early EMT.

Migration - Scratch Assays

Scratch assays were carried out to look for increased cell motility. HaCaT, TYS and TR146 cell lines were grown to confluency; the monolayer was scratched with a blunt pipette tip, and the cells were monitored at 3 hours, 6 hours and 24 hours. Migration was quantified by looking at the amount of contacts where cells had moved into the scratch. Table 9 shows the scale used and examples of the histological appearance.

0%	No closure			
20%	<10 contacts			
40%	10-30 contacts			
60%	More area in contact than not			
80%	Little evidence of the scratch remains			
100%	No evidence of the scratch remains	**TYS SF 0hr (0%)**	**TYS SF 6hr (40%)**	**TYS SF 24 hr (100%)**
		Table 9. Scale to quantify migration by percentage (left) and light microscopy images to show examples of a scratch, a contact and complete obliteration of the scratch (above).		

All of the epithelial lines migrated into the scratch to some extent. Table 10 shows the migration according to the scale above. HaCaT showed some contacts at 24 hours but there was no difference whether HA was present or not. Similarly, TYS migrated into the scratch consistently and faster than HaCaT, but the addition of HA had no effect. TR146 migrated into the space consistently, even in the negative control, but this was noticeably faster than both TYS and HaCaT. Also, the addition of HA did increase the number of contacts seen at 3 hours and 6 hours.

Sample		Average Migration (%)		
Cell Line	Added HA	3 Hours	6 Hours	24 Hours
HaCaT	-	0	0	30
	10ng	0	0	20
	100ng	0	0	30
	1µg	0	0	40
	10µg	0	0	40
TYS	-	0	40	100
	10ng	0	40	100
	100ng	0	60	100
	1µg	0	60	100
	10µg	0	40	100
TR146	-	10	50	100
	10ng	30	70	100
	100ng	30	60	100
	1µg	70	90	100
	10µg	30	80	100

Table 10. Average results of scratch assays run in duplicate. SCC cell lines TYS and TR146 migrated quicker than HaCaT. The addition of HA sped up the migration in TR146, but the effect on HaCaT and TYS was far less pronounced.

Chapter 4 Discussion

Aims and Objectives

This project aimed to identify the mechanisms and implications of increased HA synthesis in SCC. In order to identify the mechanisms of synthesis, this project has looked at which cell type is responsible, which HAS enzymes and which growth factors. As for the implications of HA synthesis, the literature suggests a link with EMT and migration, so this has been the focus of the second part of the project.

A Note on Cell Lines

Tumorigenesis is a hugely varied process, involving multiple mutations. These will involve different genes and have different causes, including both genetic and environmental risk factors, but ultimately lead to the same pathology. Such convergent mutations have been described in SCC previously (Boyle, et al., 2001) (India Project Team of the International Cancer Genome Consortium. , n.d.). For this reason, each carcinoma develops uniquely and we cannot expect cell lines from different tumours to behave exactly the same. The PM1 cell line is taken from a dysplastic lesion from forehead skin and has consistently behaved differently to the TYS and TR146 cell lines. This could be due to its epidermal rather than mucosal origin. Alternatively, this may be because it is only dysplastic and still at an early stage in tumorigenesis; or perhaps if left untreated, this lesion was never going to become neoplastic. However, since its unusual characteristics cause us to question whether it is even epithelial, it will not be discussed further in this project.

TYS and TR146 are both from well-differentiated, aggressive SCC, and both are metastatic. However, there have been many differences in behaviour, demonstrating how diverse mutations have led to similar phenotypes.

Distribution of HA Synthesis

It is well established that HA is synthesised at all surfaces of the cell membrane, and this was demonstrated again here in ICC experiments. All ICC samples stained for *HAS2* and *HAS3*, whether fibroblast or epithelial, tumour-associated or not.

Regarding distribution within the tumour, it would seem fibroblasts and some neoplastic epithelial cells are responsible for the increase in HA. The ICC results would suggest that this is primarily due to an increase in *HAS2* on addition of exogenous EGF to the cells. *In vivo*, fibroblasts are responsible for stromal regulation. However, the assays carried out here showed similar levels of HA are produced by the epithelial cell lines. This may be a result of *in vitro* growth conditions where there is no stroma; epithelial cells have to form a basement membrane for themselves. It has been reported that epithelial cultures *in vitro* have lower expression of terminal differentiation markers and high expression of basal cell keratins (Freshney & Freshney, 2004). Perhaps an increase in HA output is also a characteristic of epithelial cells in culture.

Mechanism of Increased HA Synthesis

According to the ICC results, EGF caused an increase in *HAS2* and *HAS3* in HaCaT and TR146 cells, but it was TGFβ1 that gave a similar response in TYS. Furthermore, the HA assay revealed that an increase in HA occurred on addition of EGF in TYS and TR146 but not HaCaT. This would suggest that HA expression is enhanced in transformed epithelial cells, but that different signalling pathways are affecting the different tumour cell lines. This would be an example of convergent mutations as described earlier.

It has been reported that VEGF only causes increased motility in oral SCC and not in normal fibroblast controls by phosphorylating Akt and activating the Akt pathway (Islam, et al., 2014). The EGFR family is known to activate the Akt pathway amongst others (see Figure 7). One possible model is that EGF, TGF and VEGF are activating the Akt pathway, triggering increased HA synthesis and consequently cell motility.

Since HaCaT shows an increase in *HAS2*/3 but a decrease in HA production, there must be additional levels of regulation. This may be for example decreased enzyme activity due to lack of phosphorylation or dimerisation; decreased availability of substrate; or an increase in turnover of HA by Hyals in the ECM. The literature reports that the expression of Hyals (Franzmann, 2003), MT-MMP1 (Nakamura, 2004) and ADAM proteases (which degrade CD44) is increased in head and neck SCC. However, it has been reported that more advanced tumours show decreased and/or irregular HA expression. Therefore, any model of HA regulation in the tumour must be dynamic, changing at different stages of tumorigenesis.

HA synthesis increased in both MM1 and COMD lines on addition of low levels of growth factors, but not high levels. This differential response could explain why positive correlations in *HAS2*/3 synthesis were not seen in the ICC results. The signalling via EGF and VEGF pathways are likely to be normal signalling responses usually seen during wound healing.

Implications of Increased HA Synthesis

Successful EMT determines whether a cell experiences increased motility or gains the capacity for tissue invasion (Martin, et al., 2013). It requires the loss of E-cadherin, making the cell independent from contact growth inhibition. This is followed by the expression of matrix metalloproteinases, which allow the cell to break down stromal proteins and

membranes and increase adhesion. Only two markers were used here to demonstrate EMT. A positive result would require the loss of E-cadherin expression and a gain in vimentin. None of the cell lines showed this. Vimentin expression was switched on in TR146 and correlated with increasing HA levels. However, it retained E-Cadherin expression. This result may signify an early stage in EMT but it is not conclusive. Studies of EMT in SCC associate it with cell-cell dissociation and cell movement (Grille, 2003) (Boyer, et al., 2000). Since the Western blots were inconclusive, migration assays were run to look for evidence of EMT from its effects.

Migration is movement on 2D surfaces without any obstructive stroma, as opposed to invasion, which involves the breakdown of the ECM (Kramer, 2013). A scratch assay shows collective 2D migration of epithelial sheets by means of EMT, and all epithelial cell lines were capable of this. HA signalling and motility is an important pathway in wound healing and angiogenesis, so this response may be normal physiology. However, TYS and TR146 showed increased motility compared to HaCaT in the scratch assay. Furthermore, TR146 migration was faster on addition of HA in increasing concentrations. Therefore, it is possible that HA might be involved in enhancing migration in some tumours.

However, metastasis also requires invasive capabilities. A Boyden assay would show single-cell 2D migration by means of chemotaxis through a membrane. The planned assay coated the membrane with collagen, so that a positive result would demonstrate invasion rather than simply migration (Kramer, 2013). The Boyden assays were abandoned due to the limited timespan of this project and since epithelial cell lines did not migrate well in work carried out by other researchers in the lab. However, this negative result may be significant

as this would indicate the cells have not gained invasive capabilities in the presence of HA signalling. More work needs to be carried out in this area to determine if this is the case.

Limitations and Improvements

By including epithelial and fibroblast cells, this project would look for heterotypic signalling. While these are the cell types most likely to be involved in HA signalling, there are other cell types found within the SCC that have not been considered, such as endothelial cells.

The variety and quantity of growth factors present in the tumour microenvironment may be other areas of contention. *In vivo* no growth factor is present in isolation and the growth factors used in this study may have synergistic or contradictory effects. Further experiments using a test medium containing multiple growth factors may be more representative. Also, many of the ICC results showed differential effects on *HAS2*/3 expression at different quantities of growth factors. It may be helpful to measure the amount of growth factors produced by the different cell types during normal growth phases. This could then be used as a guide as to how much growth factor is present in the SCC. Also, only EGF, TGFβ1 and VEGF were used. Other growth factors such as PDGF and FGF-2 may also affect SCC signalling pathways.

An obvious limitation of the study is that experiments were only run in duplicate. In the ICC results where the data was rarely statistically significant, extra data would make the conclusions drawn more reliable.

ICC is an excellent method to localise proteins within a cell. However, its use for the quantification of proteins is not ideal. Many factors such as the confluency of samples and variations in incubation times and washes, will affect fluorescence intensity. This becomes particularly significant where fluorescence is very low or very high. While measures were

taken to normalise the data as much as possible, it will never be as accurate a quantification as a biochemical assay.

When studying HA signalling, the most striking variable is the effect of HA chain length on signalling outcomes. The HA quantification assay used here detects HA of any length, but it would have been interesting to see which chain lengths were produced and if this changed on addition of growth factors. If the theory presented here is correct and HA is subject to increased turnover, perhaps a higher level of short chain HA fragments could be detected.

Furthermore, when looking at the implications of increased HA levels, only a medium length chain HA was added to the media. This could be inappropriate if it were found that more short chain HA is produced in SCC. Since long chain HA is associated with tissue stability and cell integrity, and short chain HA is associated with inflammatory response and migration, it could be that the inappropriate addition of long chain HA is inhibiting migration.

Conclusions

The model proposed in this study is that EGF (and in some tumours TGFβ1) causes an increase in *HAS2* expression in fibroblasts and transformed epithelial cells. A consequence of this is increased synthesis of HA and this is linked to the early signs of EMT and enhanced migration. Although this model is true of TR146, TYS behaved very differently. It is a possible explanation for the increase in SCC HA levels reported in the literature, but it is certainly not a consistent relationship in SCC.

Further work in this field would include determining whether growth factors affect the production of HA proteases, exploring the role of different size HA chains in the tumour microenvironment; and to investigate invasive abilities of the epithelial cell lines using Boyden's or similar assays.

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Appendices

Appendix 1 – Cell Culture Reagents

10% FCS, 1% L-Glutamine MEM

Foetal Calf Serum (Foetal Bovine Serum; Product 10270 Invitrogen; Product 41Q4025K Gibco) heat treated at 60°c for 30-40min.

Minimum Essential Medium Eagle 10x (MEM) (Product M-0275 Sigma):

- 150ml penicillin- streptomycin stabilised (Product P-4333 Sigma)
- 150ml MEM non-essential amino acid solution (Product M-7145 Sigma)
- 150ml sodium pyruvate (Product S-8636 Sigma)
- 33g sodium bicarbonate (Product S-8761Sigma)

L-Glutamine 200mM stock (Product G-7513; Sigma)

Medium Variations

Additives:

10mM β-Glycerophosphate	(Product G6251 Sigma)
100nM dexamethasone	(Product D1756 Sigma)
50µg ascorbic acid	(Product A7506 Sigma)

Appendix 2 – ICC Antibodies

HAS2 (H-60), Santa Cruz	Cat No: Sc-66916	Lot No: G0109 Rabbit Polyclonal
HAS3 (H-60), Santa Cruz	Cat No: Sc-66917	Lot No: K2509 Rabbit Polyclonal

Anti-Rabbit IgG conjugated with Alex Fluor 488, Cell Signaling Technology

Cat No: #4412

Appendix 3 – Statistical Formulae

Corrected Total Cell Fluorescence (CTCF)

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

Appendix 4 – ELISA Materials

DuoSet Assay Development kit for Hyaluronan (#DY3614, R and D Systems)

Wash Buffer:	PBS + 0.05% (v/v) Tween 20 (50µl per 100ml or 500µl of 10% Tween 20 per 100ml) - filtered
Blocking Buffer:	PBS + 5.0% (v/v) Tween 20 (5ml per 100ml) + 0.05% Sodium azide (50mg per 100ml) - filtered
Reagent Diluent:	PBS + 5.0% (v/v) Tween 20 (5ml per 100ml) – filtered
Stop Solution:	2N Sulphuric acid.
Substrate solution:	1:1 mixture of colour reagent A (hydrogen peroxide) and B (tetramethylbenzidine)
Streptavidin-HRP:	
Capture reagent:	0.5µg/ml Aggrecan (#842162) in PBS
Detection reagent:	0.4µg/ml Aggrecan (#842163) in Reagent Diluent
Standard Hyaluronan:	HA (#842164)

Appendix 5 – SDS PAGE and Western Blot Buffer Solutions

RIPA buffer with Protease Inhibitors: 50mM Tris HCl pH 7.2,

150mM NaCl

0.1% (w/v) SDS

1.0% (v/v) Triton x100 or 1% (v/v) NP40

1.0% (w/v) sodium deoxycholate

5mM EDTA

Laemmli Loading Buffer: 62.5 mM Tris HCl pH 6.8

2% ((w/v)) SDS

25% ((v/v)) Glycerol

0.01% ((w/v)) Bromophenol Blue

5% ((v/v)) 2-Mercaptoethanol

Laemmli Running Buffer (TGS): 25 mM Tris HCl pH 8.3

192 mM Glycine

0.1% ((w/v)) SDS

Towbin Transfer Buffer: 5.82g Tris

2.93g Glycine

3.75ml 10% ((w/v)) SDS

200ml Methanol

to 1litre with dH2O

Blocking Buffer: 100ml 1 x TBST

1g Dried milk powder

10 x TBST: 24.2g Tris

80g Sodium chloride

5ml Tween 20

14ml Hydrochloric acid (adjust to pH 7.6)

to a total volume of 1 litre with dH2O

10x TBS: as TBST without Tween 20